THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXIV JULY-AUGUST, 1958 NUMBER 4

DISSEMINATED LUPUS ERYTHEMATOSUS, SCLERODERMA, AND DERMATOMYOSITIS AS MANIFESTATIONS OF SENSITIZATION TO DNA-PROTEIN

I. AN IMMUNOHISTOCHEMICAL APPROACH *

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The semantic concepts of "connective tissue disease" and "collagen disease," proposed respectively by Klinge[1] and by Klemperer, Pollack and Baehr,[2] have stimulated many investigations of the normal and pathologic physiology of the connective tissues and have contributed significantly to the body of knowledge in this confusing area. In the past, however, attention has been focused primarily upon the late stages of the "collagen diseases" and their structural manifestations, notably fibrinoid degeneration or necrosis, and cicatrization. Disseminated lupus erythematosus, scleroderma, and dermatomyositis, little understood members of the group, have been conspicuous subjects for retrospective analysis.

The present investigation rests upon an examination and attempted elucidation of certain apparent antecedents to the fully developed lesions seen in these diseases. Although the evidence bearing upon the nature of collagen disease has been largely hypothetical, a basis in hypersensitivity has been implied sufficiently often to warrant further attention. Indeed, the recent work of Haserick, Lewis and Bortz,[3] incriminating gamma globulin in the development of the LE cell phenomenon, has provided a substantial spur in the same direction. The major premise of the present inquiry has been derived from the well known association between circulating antibodies and the gamma globulin fraction of serum protein. The possibility that collagen diseases, and particularly those mentioned above, might follow a hyperimmune reaction, has prompted an exploration of serums from patients

* Supported by U.S. Public Health Service, Grant C-2451.
Received for publication, November 22, 1957.

afflicted with the specified disorders for the presence of specific antibody.

Gamma globulin and raw serums from individuals with various so-called collagen diseases have been employed as histochemical stains against autologous, homologous, and heterologous tissues, by the fluorescent antibody techniques developed by Coons and associates.[4-7] A binding by tissue components has been taken as presumptive evidence of local antigen-antibody precipitation, reflecting the presence of either true circulating antibody or an entity possessing similar traits within the original test serum.

Preliminary experimental results have revealed a bizarre but nonetheless reproducible reaction between cellular nuclei in human and animal tissues and an active agent seemingly present in the serum gamma globulin fractions of patients with disseminated lupus erythematosus (DLE), scleroderma (SCL), and dermatomyositis (DMS), as well as in cases of clinical rheumatoid arthritis presenting positive LE cell tests. Positive reactions have been characterized by a staining of nuclear material by test serum globulin labeled with fluorescent dye, when viewed in a fluorescence microscope under ultraviolet light. The specific nature of this hitherto unreported histochemical localization is not wholly clear, but some of the more provocative interpretations are discussed below and related to problems of pathogenesis and clinical application. Recently, we have become aware of similar investigations in progress elsewhere; only abstracts are at hand, however, and a detailed comparison of data must, therefore, be postponed.[8,9]

Materials and Methods

Clinical Material

Serums. Forty-three samples of serum were collected from 38 selected patients at the following institutions: Robert Breck Brigham Hospital (R.B.B.H); Boston Lying-in Hospital (B.L.I.H.); House of the Good Samaritan (H.G.S.); Children's Medical Center; Massachusetts General Hospital (M.G.H.); and Veterans Administration Hospital at West Roxbury, Massachusetts (V.A.H.). Within the collagen disease group, specimens were obtained from patients with acute disseminated lupus erythematosus (5 cases), discoid lupus (1 case), scleroderma (6 cases), dermatomyositis (2 cases), rheumatoid arthritis (8 cases), temporal arteritis (2 cases), and glomerulonephritis (1 case). A single serum specimen from a patient suffering from a penicillin reaction was also examined. Controls included serum from young and old individuals of both sexes, normal women in early and late stages of pregnancy, and patients with pre-eclamptic toxemia, acute

TABLE I
Synopsis of Staining Reactions

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Patient*	Diagnosis	Source	LE prep.	Tissues: Human: Skin Dir.	Skin Indir.	Placenta Dir.	Placenta Indir.	Breast	Rabbit: Kidney	Myocardium	Lung	Adrenal
SCL Ia	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	o	o	o	o	o
SCL Ib	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	−	+	−	−	o
SCL Ic	Scleroderma, eclampsia	B.L.I.H.	−	+	+	+	+	+	+	−	−	+
SCL Id	Scleroderma, eclampsia	R.B.B.H.	−	o	+	o	+	o	−	±	−	−
SCL IIa	Scleroderma	R.B.B.H.	−	o	+	o	+	+	+	+	+	+
SCL IIb	Scleroderma	R.B.B.H.	−	+	+	+	+	o	+	+	+	+
SCL III	Acrosclerosis	R.B.B.H.	−	o	+	o	+	−	+	+	+	−
SCL IV	Scleroderma	Private	−	o	+	o	+	o	±	±	+	+
SCL V	Atypical scleroderma	R.B.B.H.	−	o	−	o	−	o	−	−	−	−
SCL VI	Inactive scleroderma	R.B.B.H.	−	o	±	o	−	−	−	−	−	−
DLE I	D. L. E.	R.B.B.H.	+	+	+	+	+	o	+	+	+	+
DLE II	D. L. E.	R.B.B.H.	+	o	+	o	+	+	−	+	−	+
DLE III	D. L. E.	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
DLE IV	D. L. E.	H.G.S.	+	o	+	o	+	o	+	+	+	+
DLE V	D. L. E.	H.G.S.	+	o	+	o	+	o	+	+	+	+
DLE VI	Discoid L. E.	B.L.I.H.	−	o	−	o	−	−	−	−	−	−
DMS I	Dermatomyositis	Private	o	o	+	o	+	o	+	+	+	+
DMS II	Dermatomyositis, inactive	R.B.B.H.	o	o	−	o	−	−	−	−	−	±
RhA Ia	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA Ib	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA II	Rheumatoid arthritis	R.B.B.H.	−	o	−	o	−	−	−	−	−	−
RhA III	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	−	−	−	±	−	−
RhA IV	Rheumatoid arthritis	R.B.B.H.	−	o	+	o	−	−	−	−	−	±
RhA V	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
RhA VI	Rheumatoid arthritis	R.B.B.H.	−	o	+	o	+	o	+	+	+	+
RhA VII	Rheumatoid arthritis	R.B.B.H.	+	o	−	o	−	o	−	−	−	−
RhA VIII	Rheumatoid arthritis	R.B.B.H.	+	o	+	o	+	o	+	+	+	+
TA I	Temporal arteritis	M.G.H.	o	o	−	o	o	o	−	−	−	−
TA II	Temporal arteritis	M.G.H.	o	o	−	o	o	o	−	+	−	−
GN I	Glomerulonephritis	V.A.H.	o	o	−	o	−	−	−	−	−	−
PR I	Penicillin reaction	V.A.H.	o	o	−	o	−	−	−	−	−	−
Controls	12 cases including instances of hydatidiform mole, toxemia, eclampsia, normal pregnancies, other male and female patients of various ages					Includes 4 conjugates for direct staining Results uniformly negative						

Key: + Nuclei stained
− Nuclei not stained
± Weak or irregular stain
o Not performed

*Patients coded by literal abbreviations for clinical diagnosis, followed by Roman numerals assigned in chronological order. Lower case letters represent successive serum samples from same patient.

eclampsia, and hydatidiform mole. A detailed listing appears in Table I. Each case was classified according to prevailing clinical and pathologic diagnostic standards, and most patients were seen personally by one or another of the authors.

Blood drawn from each patient under sterile conditions was reduced to serum, distributed in small aliquots, quick-frozen in an acetone-dry ice mixture, and stored at −17° C. until required.

Tissues. Fresh surgical specimens, representing a variety of normal human tissues, were secured at the Robert Breck Brigham, Peter Bent Brigham, and Boston Lying-in Hospitals, and at the Children's Medical Center. Fresh samples of rabbit tissue, including the major organ systems, were used in addition. Necropsy material, although employed in the initial phases of the study, was found to be unsatisfactory. Each portion of tissue, as excised, was placed in a test tube and quick-frozen. Samples were stored in a deep freeze unit, and hardened over night in dry ice before cutting.

Preparation of Serum Conjugates

Globulin Fractionation. Serums were separated into crude globulin fractions at 0° C., with gentle mechanical stirring, according to the procedure of Coons.[10] An initial sample, generally about 20 to 40 ml., was diluted with an equal volume of normal saline. Saturated ammonium sulfate was added drop by drop with stirring, yielding a solution half saturated with respect to the precipitant. The turbid protein suspension was stirred for some time, and then stored over night in the cold to permit the reaction to reach completion. After centrifugation and washing with half-saturated ammonium sulfate, the globulin was dissolved in saline to approximately one third of the original volume, and then dialyzed in the cold against saline buffer[4] to remove both sulfate and ammonium nitrogen. The opalescent protein solution was protected with merthiolate in a final concentration of 1:10,000 and stored at 4° C.

Conjugates. The globulin fractions were conjugated to fluorescein isocyanate as described elsewhere.[4] Absorption, as performed in the classical procedure, was omitted to prevent removal of active principle by tissue nuclei. Globulin fractions and conjugates were spot-checked for purity by paper-strip electrophoresis; patterns indicated concentration in the gamma globulin region, with only traces of albumin. The latter did not interfere perceptibly with later procedures. Conjugates against human albumin (AHA; supplied by Dr. D. Gitlin, Children's Medical Center, Boston, Massachusetts) and gamma globulin (AHG; supplied by Dr. C. Liu, Department of Bacteriology and Immunology,

Harvard Medical School, Boston, Massachusetts) were prepared by active immunization of rabbits, while that formed against rabbit gamma globulin (ARG; supplied by Dr. B. K. Watson, Department of Bacteriology and Immunology, Harvard Medical School) utilized goat antiserum. The fluorescent conjugates used in direct staining procedures are catalogued in Table II.

TABLE II

Fluorescent Conjugates for Direct Staining

Code	Patient	Diagnosis
SCL Ia-F	SCL I	Scleroderma; eclampsia 2 mos. post partum
SCL Ib-F	SCL I	Scleroderma; eclampsia, acute
SCL Ic-F	SCL I	Scleroderma; eclampsia 5 mos. post partum
SCL IIb-F	SCL II	Scleroderma
DLE I-F	DLE I	Disseminated lupus erythematosus
C I-F	C I	Control; early normal pregnancy
C II-F	C II	Control; term normal pregnancy
C III-F	C III	Control; pre-eclamptic toxemia
AHG		Rabbit anti-human gamma globulin
AHA		Rabbit anti-human albumin

Cases other than those listed here have been investigated by indirect technique. For any serum specimen, the corresponding conjugate prepared for direct staining reactions has been designated by an upper case "F" following the serum sample number: SCL IIb-F.

Tissue Preparation

Sectioning. Frozen and prehardened blocks were cut at 5 μ in a cryostat at −17° C. After thawing on a fingertip and air drying at room temperature under a fan, the unstained sections were stored in the cold.

Fixation. Most stains were performed upon unfixed material. Other tissues were fixed in 5 per cent formalin or 95 per cent ethanol for 5 minutes at 37° C. Absolute methanol and anhydrous acetone, 10 minutes at 37° C., were also used.

Histochemical Procedures

Staining. Although unstained material was set aside occasionally for as long as 3 days after cutting to determine stability, sections generally were stained within an hour after preparation. Both the direct and the indirect or layering methods were used.

In the direct technique, tissues were stained with specific conjugates containing fluorescein-labeled gamma globulin, and local precipitation of fluorescent material was presumed to indicate sites of immune reaction. When fixed, tissues were dried in air at 37° C. for 30 minutes. After a 5 minute wash in saline buffer at room temperature, each slide

was laid flat and a drop of the proper conjugate spread over the tissue. The section was incubated with tagged protein for 20 minutes, whereupon the stained slides were washed once again in buffer for 10 minutes, to elute uncombined conjugate. A clean cover slip was mounted on each slide with a drop of buffered glycerol.[4]

The indirect method consisted of bathing the tissue with unconjugated serum, washing, and then staining with labeled anti-gamma globulin antiserum to localize any specific antibody precipitated from the serum during the first step. Because of a probable multiplication of binding sites when fixed antibody serves in turn as antigen, this technique is considered somewhat more sensitive.[7] One drop of the serum to be evaluated for antibody content was incubated with the tissue under a humidifier for 20 minutes, whereupon the section was washed 5 minutes in saline buffer to remove uncombined protein, and then stained for 30 minutes with a conjugate against gamma globulin. The section was washed once again in saline buffer for 5 minutes, mounted in buffered glycerol, and examined. When human serums were tested, the sections were counterstained with conjugated rabbit anti-human gamma globulin antiserum globulin (AHG), with conjugates AHA and ARG as controls.

Antigen Removal Tests. Specific enzymatic digestions were performed upon tissue sections; in most instances, a drop of enzyme solution was incubated on the tissue for 1 to 5 minutes at room temperature, followed by washing with saline buffer. Crystalline deoxyribonuclease (Nutritional Biochemicals Corp., Cleveland, Ohio) was used at concentrations of 0.01 to 1.0 mg. per ml. of buffer at pH 7.1. Crystalline bovine ribonuclease (Worthington Biochemical Corp., Freehold, New Jersey) was employed at a maximum concentration of 1.0 mg. per ml. Both enzymes were made up in the special phosphate-carbonate buffer* recommended by Wilbur and Anderson.[11,12]

Light trypsinization of the tissues was carried out to yield either destruction of the reactant or enhancement of the reaction; the latter possibility was suggested by the work of Coffin and Pickles,[13] who demonstrated the ability of trypsin to restore the specific agglutinating capacity of periodate-treated erythrocytes. Trypsin, as a solution of Viokase P-130D diluted to 1:100, was incubated with the tissue at 37° C. for varying periods up to 15 minutes. The results of the several digestions are shown in Table III.

* Composition of special phosphate-carbonate buffer as follows:

KH_2PO_4 0.0094 M	$NaHCO_3$ 0.0015 M
K_2HPO_4 0.0125 M	Sucrose 0.145 M

Specific gravity at 25°C.: 1.023. Slightly hypotonic: 0.7 X isotonic. pH 7.1.

TABLE III

Antigen Removal Tests
(Tissue + Reagent + Stain)

Reagent	Stain*			
	Conjugates		Serums	
	SCL IIb-F	DLE I-F	SCL II	DLE I
Formalin: 5% for 5 min.	+	+		
Ethanol: 95% for 10 min.	+	+	+	+
Acetone, anhydrous, 10 min.	+	+	+	+
Heating tissue 56° for 30 min.				
Wet chamber	−	−		
Wet chamber, heat conj.	−	−		
Dry chamber	+	+		
Dry chamber, heat conj.	+	+		
RNASE: 1 mg./ml. for 5 min.	+	+	+	+
DNASE:				
>0.05 mg./ml. for 1 min.	−	−	−	−
<0.01 mg./ml. for 1 min.	+	+	+	+
Trypsin:				
1 to 5 min.	+	+	+	+
5 to 15† min.	−	−	−	−
Saline buffer, 2 hr. wash before staining	+	+		

Key: + Nuclei stained
— Nuclei not stained
* Conjugates used direct; serums used indirect.
† Tissues destroyed by prolonged trypsinization.

TABLE IV

Inhibition Procedures
(Tissue + Serum + Conjugate)

Serum	Skin tissue		Kidney tissue	
	Conjugate		Conjugate	
	DLE I-F	SCL IIb-F	DLE I-F	SCL IIb-F
SCL IIa	+	−	±	±
SCL III	+	−	±	+
SCL IV	+	+	±	±
DLE I	−	+	−	+
DLE II	−	+	−	+
DLE III	+	+	−	+
DLE IV	−	+	−	+
DLE V	−	+	±	+
DMS I	+	+	+	+
Controls	−	−	−	−
Gamma globulin fractions				
SCL IIa			+	+
DLE I			−	+
DLE III			+	+

Key: + Nuclei stained
— Nuclei not stained
± Faint irregular staining

Immunologic Inhibitions. The specificity of histochemical binding was explored in a number of inhibition procedures, as represented in Table IV. In direct inhibition tests, or so-called auto-inhibitions, the tissue was treated first with an unconjugated serum, and then with the conjugate of the same serum; abolition of staining was presumed to be due to a blockade of binding sites by the raw serum. Cross inhibition procedures consisted of attempted staining of a tissue by the conjugate of a serum following prior exposure of the section to unconjugated serum from a different patient.

Microscopy

After staining, sections were inspected by ultraviolet fluorescence microscopy. Standard monocular microscopes with dark-field condensers were illuminated by automatic feed carbon arcs. Visible light was screened out by a copper sulfate cell and Corning No. 5840 filter, while stray ultraviolet was removed at the ocular by a disk of Euphos glass. Specimens were inspected at magnifications of 80, 200, and 400 times. The underside of the slide was sealed to the condenser with a drop of nonfluorescent immersion oil.

Photography

All typical reactions were recorded in both monochrome and color, using twin Leica 35 mm. cameras with Microipso attachments, the oculars of which contained Euphos filters. Black-and-white photographs were made on Kodak Tri-X film, exposed 4 to 8 minutes depending upon image intensity. These were developed commercially. For color, Anscochrome daylight emulsion was exposed 8 to 10 minutes and processed to an ASA rating of 200, rather than at the usual speed of 32. The stage coordinates for each frame were recorded, and the slide was later counterstained with hematoxylin and eosin for topographic correlation.

Results

Staining Reactions

Various autologous, homologous, and heterologous tissues were stained by both the direct and indirect methods. In every case the histochemical agent was protein labeled with fluorescein isocyanate. In all 5 cases of DLE, in 4 of 6 patients with SCL, and in the single case of active DMS examined, tissue nuclei were stained intensely, fluorescing as a brilliant chartreuse green under ultraviolet light. Intranuclear localization was confirmed by subsequent hematoxylin and eosin counterstaining of the same slides, as illustrated in Figure 10.

In this report, the term "positive reaction" implies such binding of protein by intranuclear material.

The two negative cases of SCL were clinically inactive. Irregular positive staining was observed in some of the serums from patients with rheumatoid arthritis, being especially marked in those demonstrating a positive LE cell phenomenon. The reaction was negative in cases of temporal arteritis, glomerulonephritis, the case of penicillin sensitivity, and in the dozen control patients.

The direct and indirect techniques were mutually confirmatory; a given test serum was positive by both methods or not at all. Nuclei generally were stained more intensely by the indirect or layering technique. It should be noted, however, that sections stained directly showed localization only within the nuclei of tissue cells or, occasionally, in sites of focal necrosis; the background was unstained and dark, save for intrinsic autofluorescence. With the indirect technique, the final staining with anti-gamma globulin produced a generalized histochemical localization of this protein, especially in vascular lumens and extracellular interstices. Positive nuclear reactions, fortunately, were found only with serums positive by the direct stain, and never after direct anti-globulin staining alone, so that indiscriminate labeling of tissue globulin introduced no ambiguity.

Although it is customary to absorb fluorescent conjugates against desiccated tissue powder preparations, this step was omitted to prevent interference with the reactions by exposure of the reagents to intranuclear material. Indeed, binding was impaired or inhibited altogether by prior absorption. Miescher and Fauconnet,[14] it may be noted, were able to absorb the factor involved in the LE cell phenomenon, using isolated cell nuclei.

A curious inverse relation between nuclear and cytoplasmic staining was observed occasionally. In vigorously positive reactions, nuclei alone appeared to bind conjugate; with weak or negative reactions, however, a faint staining of cytoplasm was seen. Typical positive and negative reactions are shown in Figures 1 to 3 inclusive.

Effect of Tissue Fixation and Aging

Positive reactions were not prevented by fixation of the tissue in formalin, acetone, or alcohol. Although autofluorescence increased steadily with tissue aging, the nuclear substance operating in the reactions appeared stable in this respect; successful intranuclear binding has been found in sections kept at 4 to 5° C. as long as 7 days after cutting. Tissue blocks have been stored at least 18 months in a dry ice

refrigerator with no impairment of nuclear reactivity. In the present investigation, however, sections, as a rule, were stained within an hour after cutting, and were unfixed unless otherwise noted.

Correlation with the LE Cell Preparation

As shown in Table I, positive nuclear binding of gamma globulin generally paralleled positive LE preparations with serum from the same patient. The converse, however, did not hold, for a negative LE cell test did not preclude a positive reaction with nuclei.

Effect of Heat and Moisture

Heating the conjugate and tissue slices at 56° C. for 30 minutes did not prevent a subsequent positive reaction, if the tissue remained dry. Some sections were heated dry up to 65° C. for 30 minutes with no apparent ill effect. Warming in a humid atmosphere, or after direct moistening of the section, blocked the reaction.

Role of Autolyzed Protein

Frequently gamma globulin from cases of DLE, SCL, and DMS reacted with pyknotic cells or necrotic tissue constituents, with a resultant fluorescence of apparent nuclear debris. To determine whether the type of positive staining reaction under examination in the present study reflected haphazard binding with autolyzed protein, frozen sections were prepared from homotransplants of rabbit liver 12 days following the surgical procedure and treated with conjugates against both albumin and globulin. No staining whatever was observed in nuclear sites, or in the necrotic central portions of the grafts. Only the cytoplasm of peripherally disposed cells was stained, evidently because of a passive centripetal diffusion of host serum protein.

Chemical Aspects of the Nuclear Binding Reaction

Role of Serum Proteins. The preceding experimental data, particularly the results of indirect reactions, strongly suggest that the active serum principle is, or shares in the properties of, an abnormal gamma globulin. To ascertain the connection, if any, between serum protein and the reactive substances within cell nuclei, all tissues studied were surveyed topographically for albumin and gamma globulin by the use of specific antiserum conjugates. All stains were direct. Since nuclei consistently failed to be stained, they were presumed to lack significant concentrations of indigenous albumin or gamma globulin; hence the reactions probably were not mediated by the presence of these protein fractions within tissue nuclei. It is well to note that on the basis of the

indirect stain reactions performed here, as well as various protein localizations to be reported elsewhere, the anti-blood protein conjugates employed in this study appeared to stain both normal and abnormal serum proteins alike.

Role of Deoxyribonucleic Acid and Ribonucleic Acid. The participation of nucleoprotein was investigated by enzymatic digestions with specific nucleoproteinases. The staining reaction was inhibited by prior exposure of the tissue to deoxyribonuclease for 1 minute, at concentrations of 0.05 mg. per ml. or more, but not by incubation with the same enzyme for 2 minutes at 0.01 mg. per ml. or greater dilutions. At practically all concentration levels, occasional nuclei were nonreactive, but a gradually increasing inhibition was observed as the level of deoxyribonuclease was raised (Figs. 4 and 5). Ribonuclease, on the other hand, failed to prevent a positive reaction, as shown in Figure 6, even at a strength of 1.0 mg. per ml. for 5 minutes. The same buffer in which the nucleases were dissolved did not inhibit the reaction after a 2 hour contact with the tissue.

Effect of Tryptic Digestion. Crude trypsin (Viokase) was used upon sections of skin and placenta, in a dilution of 1:100, and at varying intervals up to a maximum of 15 minutes. Within the first 5 minutes of digestion, the cytoplasm of cells was destroyed, leaving behind free nuclei which nevertheless showed a bright staining reaction. The stain was abolished completely by the end of 15 minutes. In the case of skin, the cytoplasm of epithelial cells was digested away, there remaining isolated faint and shadowy remnants of nuclei, which appeared slightly autofluorescent. Placenta was destroyed almost entirely, save for scattered leukocytes which may have survived because of intrinsic antitryptic defense mechanisms. These cells appeared unaffected by trypsin and indeed were stained by the labeled abnormal gamma globulins.

Serum Inhibition Reactions

Auto-inhibition. In every instance, nuclear binding was blocked wholly when a tissue was treated first with an unconjugated abnormal gamma globulin, and then with a fluorescent conjugate of the same serum protein (Fig. 8).

Cross-inhibition. Cross-inhibition reactions were performed among the serums from different cases of DMS, DLE, and SCL. The results are presented in Table IV. Serums from lupus patients frequently, though not invariably, showed a capacity for mutual inhibition. These serums, however, generally failed to block the binding of gamma globulins from cases of SCL. The results also varied somewhat accord-

ing to the organ tested. DMS serum, as represented by the single active case studied, failed to inhibit either DLE or SCL. A typical cross-inhibition reaction is illustrated in Figure 9.

Discussion

Immediate Implications of Experimental Findings

Patients afflicted with DLE, SCL, and DMS, and also those with rheumatoid arthritis showing a positive LE cell phenomenon, appear to carry within their serums an unusual protein constituent possessing a reactive affinity with one or more intranuclear nucleoproteins in human and animal tissues. This active serum component remains with the globulin fraction after crude chemical separation, and can be tagged specifically by fluorescent conjugates against gamma globulin. It is possible to examine the abnormal globulin of such patients with the various auto- and cross-inhibition procedures employed by Coons and Kaplan[4] as measures of immunologic specificity.

The intranuclear tissue reactant which binds abnormal gamma globulins from test patients may be digested enzymatically by deoxyribonuclease solutions above a concentration level of 0.05 mg. per ml. within two minutes, while more dilute solutions appear to affect the tissue component only incompletely or not at all. Ribonuclease, in contrast, does not alter the reaction perceptibly. The receptivity of the intranuclear factor is not abolished by prior trypsinization of the tissue sections, except at concentration levels sufficient to destroy the nuclei altogether. Fixation as described does not impair the reaction.

The gamma globulin-nucleoprotein reaction is not inhibited by heating the tissue for 30 minutes at 56° C., but it is blocked, however, when the section is warmed in a moist chamber. Reactivity is not restored by the addition of fresh guinea pig serum, indicating that complement probably is not required in the *in vitro* nuclear binding phenomenon.

Since a positive reaction may be observed after heating the tissues and conjugates to 65° C. for 30 minutes, it is not likely that the combination of abnormal gamma globulin with nucleoprotein depends upon enzymatic activity.

Thus, in the collagen diseases under investigation it would seem that affected patients possess a gamma globulin which behaves at least superficially like an antibody, and hence is designated provisionally as such in this report. This putative circulating antibody binds consistently with an intranuclear substance, involving, for its reactive identity, at least DNA and perhaps also an unspecified protein moiety. The tissue reactant is not species specific, occurring in autologous, homo-

logous, and heterologous nuclear material. On the basis of available evidence, it appears to be nucleoprotein containing DNA, and may be regarded as an antigenic substance.

Histologic Correlations

The capacity of abnormal gamma globulins to react with nuclear materials, in the opinion of the authors, is the overt expression of a fundamental disturbance common to at least DLE, SCL, and DMS, and possibly other collagen diseases as well. The nuclear binding trait does not appear limited solely to those patients with positive LE reactions; cases of SCL, among others with negative LE cell preparations, exhibited positive nuclear staining responses.

In all probability the LE cell inclusions and the basophilic bodies commonly found in DLE are interrelated phenomena. On the basis of histochemical evidence, Klemperer, Gueft, Lee, Leuchtenberger and Pollister[15] regarded the two structures as one and the same, and the finding of DNA in both has made their nuclear origin indisputable.

Alteration of the nucleus appears prerequisite to the development of the LE cell inclusions and basophilic bodies, as was demonstrated *in vitro* by Snapper and Nathan[16] in a case of DLE; little imagination is necessary to extend the same factitive process to the basophilic bodies as well. The interrelationship is strengthened further by the fact that the formation of both structures is mediated through the gamma globulin serum fraction of affected patients. Haserick and his colleagues[3] were able to duplicate the LE cell phenomenon with serums free of gamma globulin, while Gueft[17] reported the presence of unusually large amounts of protein within LE cell inclusions. Mellors, Ortega and associates[18,19] have localized gamma globulin in the inclusions by means of the fluorescent antibody technique. It may be concluded, therefore, that nuclear alteration or the formation of basophilic bodies is the initial step in a developmental sequence; this is followed by cytophagocytosis resulting in LE cells. Since both nuclei and serum gamma globulin are involved in the formation of basophilic bodies and LE cells, these agents probably also participate in the nuclear binding trait reported here; the latter effect thus may represent a broad and fundamental phenomenon related to the pathogenesis of the several diseases under investigation.

Clinicopathologic Correlations

The literature devoted to DLE is extensive, and only a few key references need be cited.[20-22] The salient clinicopathologic features have been summarized by Dubois.[23] Available information on SCL is

increasing rapidly,[24-28] and DMS, the least common of the three, has been discussed at length by Keil[29,30] and by Kinney and Maher.[31]

The question of whether DLE, SCL, and DMS reflect one and the same disease process has long been the basis for disagreement. Indications of similarities and differences have been as numerous as the authors proposing them. Recently Kampmeier[32] has submitted examples of interconversion in support of a hypothesis that all of the so-called collagen diseases are inherently interrelated. On purely clinical grounds, Beigelman, Goldner and Bayles[33] have classified DLE and SCL as expressions of a common disease mechanism which, when acute and severe, is seen as DLE, and when chronic and benign, appears as SCL. The classification of acrosclerosis is also controversial.[24,34] Because the single case studied in the present survey gave strong and reproducible nuclear binding reactions, the authors believe this curious disease may be considered a variant of SCL.

As a result of the experimental findings described here, as well as other work still in progress, it appears likely that the nuclear binding trait may reflect a natural phenomenon which is genuine, basic, and common to DLE, SCL (including acrosclerosis), DMS, and possibly a number of other diseases as well. The latter would include those cases of rheumatoid arthritis accompanied by positive LE preparations. Should these several clinical forms not be identical, they nevertheless appear to share similar properties, at least in relation to a demonstrable reactive affinity between serum gamma globulins and DNA-protein.

The binding trait can be blocked by auto-inhibition, and—at times—by cross-inhibition. The former tests were consistent, but the latter gave variable responses. In any consideration of inhibition experiments, it is well to bear in mind the reversibility of antigen-antibody combinations. The matter of concentration, not investigated thoroughly at this time, is also of great importance. It is noteworthy that a typical positive reacting serum from SCL, SCL IIb, which by itself gave constantly intense reactions, regularly failed to cross-inhibit a weaker conjugate from DLE, DLE I-F.

From the results of the various inhibition tests, one may conclude that sensitization in one case may be due to one or more DNA-proteins, while in another patient the responsible tissue reactant may be one or more antigenically distinct DNA-proteins. Regardless of the initiating agent, the common end result is the same fundamental pathologic sequence.

Pathogenetic Considerations

Since the publication of the concepts concerning connective tissue disorders by Klinge[1] and Klemperer,[2] and the inclusion of SCL in this group by Masugi and Yä-Shu,[35] these disorders have been ascribed

generally to some sort of allergic state. A basis in the immunity mechanism, however, was supported principally by superficial morphologic considerations, such as the nonspecific features of fibrinoid degeneration and hyalinization of connective tissue. Despite many other theories,[36-41] hypersensitivity remains in broadest favor, even though supporting evidence in the literature has been only fragmentary.[18,19,42,43]

In addition to the auto- and cross-inhibition tests reported here, the results of animal experimentation offer strong, although not absolute, evidence in support of a hyperimmune mechanism. In this laboratory, following the active immunization of albino rabbits against calf thymus nucleoprotein, the serum gamma globulin from these animals exhibited the same type of histochemical reaction seen in our clinical cases. There was binding not only with calf thymus nuclei, but also with the nuclei of other autologous, homologous, and heterologous tissues. The experimentally produced rabbit antiserum against nucleoprotein, moreover, showed not only auto-inhibition but also positive cross-inhibition with the serums of some patients. Details of the animal series, and of various confirmatory *in vitro* procedures, will be furnished in a subsequent report.*

It is appropriate to inquire in what manner sensitization may occur initially. No concrete answer can be offered at the present time. Relying, however, upon the experimental evidence of non-species specificity in the gamma globulin-nucleoprotein binding reaction, it is suggested that sensitization, whether immunologic or not, may begin as a response against heterologous nucleoproteins, becoming manifest thereafter as autoimmunity against one or more native intranuclear substances.

One possible pathway for the development of the disease process may be through intra-uterine sensitization against fetal or placental tissues. The first case investigated, SCL I, did not show positive nuclear staining until an episode of acute eclampsia. Indeed, this patient has related temporally her various medical problems, including SCL, to the onset of eclamptic seizures, claiming good health before that time. SCL in this patient has remained clinically active during the 18 months she has been followed, and her serum specimens have presented

* Since the preparation of the present manuscript, a pilot study has indicated that the histochemical phenomena described here may be duplicated *in vivo* by techniques based upon the general premise of hypersensitivity against nucleoprotein, suggesting the possibility of eventual production of the disease complex by artificial means. Rabbit antihistone antiserum has exhibited identical nuclear staining capacity, as well as cross inhibition with serum from a case of human scleroderma. A preliminary report has been published elsewhere (Bardawil, W. A.; Toy, B. L., and Galins, N. Hypersensitivity to histone induced experimentally in rabbits. [Preliminary communication.] *Lancet*, 1958, 1, 888–889). These findings have been confirmed by a subsequent investigation currently in progress; further details will be reported in the near future.

strong and constant positive nuclear reactions during this period.* The problem of intra-uterine sensitization in animals has been explored by Brambell, Hemmings and Henderson.[44]

The apparent sequential association between collagen disease and antecedent streptococcal infections is well known, albeit poorly understood. Swineford and Holman,[45] in 1949, reported a tendency to delayed local reactions following immunization with crude nucleoprotein extracts from a number of bacterial species, including both *Streptococcus viridans* and *Streptococcus hemolyticus*. Bacterial or viral DNA-protein, derived from invading micro-organisms, merits inclusion in a list of hypothetical heterologous antigens.

Once established, a state of sensitivity may be maintained by the small amounts of nucleoprotein liberated as cells perish spontaneously, as in physiologic necrobiosis, or are destroyed by pathologic events. Bunting[46] has described the interstitial accumulation of deoxyribonucleoprotein in areas of necrosis within a number of organs. Billingham, Brent and Medawar[47] have presented evidence that, at least in the case of reactions to skin transplantation, nucleoprotein hypersensitivity appears to be a significant factor. The same investigators have suggested that the release of nucleoprotein from living cells in the body may be a normal and constant occurrence.

Trauma may play an important role in the pathogenesis of certain lesions in a given disease, very much in the manner proposed by Massell, Mote and Jones[48,49] in the formation of subcutaneous nodules in rheumatic fever. The possible implication of some type of enzymatic activity has been studied by Schlamowitz, DeGraff, Schubert and Morrison[50-53] in connection with rheumatoid arthritis.

The elucidation of what, if any, role is played by the establishment, maintenance, and interruption of nucleoprotein hypersensitivity in the clinical course of patients affected with DLE, SCL, and DMS must await the results of further inquiry. In fact, a better understanding of the natural history of the DLE-SCL-DMS complex will follow upon improved knowledge of nucleoprotein metabolism in both normal and deranged states.

Summary

A peculiar binding trait of gamma globulin has been observed in patients with disseminated lupus erythematosus, scleroderma, and dermatomyositis. By the use of fluorescent antibody techniques, this serum factor has been shown to possess a reactive affinity with intranuclear material, probably deoxyribonucleoprotein, within autologous, homologous, and heterologous tissues. The findings are correlated and

* This patient has died 24 months after the onset of disease, with necropsy findings typical of generalized scleroderma.

prompt the suspicion that the above diseases, and perhaps related conditions such as rheumatoid arthritis, may be manifestations of a common disease process initiated by sensitization against either intrinsic or extrinsic nucleoprotein. Nuclear binding appears to require the participation of DNA but not RNA, and complement probably is not involved in the *in vitro* phenomenon. The pathogenesis of so-called diseases of collagen, as represented by those investigated, is discussed briefly.

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The authors wish to express particular appreciation to Dr. A. H. Coons, Department of Bacteriology and Immunology, Harvard Medical School, for instruction in the use of fluorescent antibody and for valuable editorial suggestions. Acknowledgments are also due Mrs. J. Clancy and Miss E. Grupp, for technical assistance. Dr. J. Homans, Jr., Brookline, Massachusetts, provided a case of DLE; Dr. W. H. Birchard, Arlington, Massachusetts, a case of DMS; and Dr. A. P. Hall, Robert Breck Brigham Hospital, various cases in the collagen disease group.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 to 9 inclusive photographed by ultraviolet light in fluorescence microscope, as described in text.

FIG. 1. Homologous positive nuclear staining of normal immature human placenta by conjugate from case SCL Ia-F. Direct technique. × 100.

FIG. 2. Heterologous positive staining of normal rabbit liver by serum from case SCL II, followed by anti-human gamma globulin conjugate AHG. Indirect technique. × 260.

FIG. 3. Typical negative nuclear reaction, by indirect technique. Normal human placenta, stained by anti-human gamma globulin conjugate AHG. × 260.

FIG. 4. Digestion of normal human placenta by deoxyribonuclease at concentration of 1.0 mg. per ml. for 3 minutes, with subsequent indirect stain by serum from case SCL II, followed by conjugate AHG. Note inhibition of nuclear binding reaction. × 260.

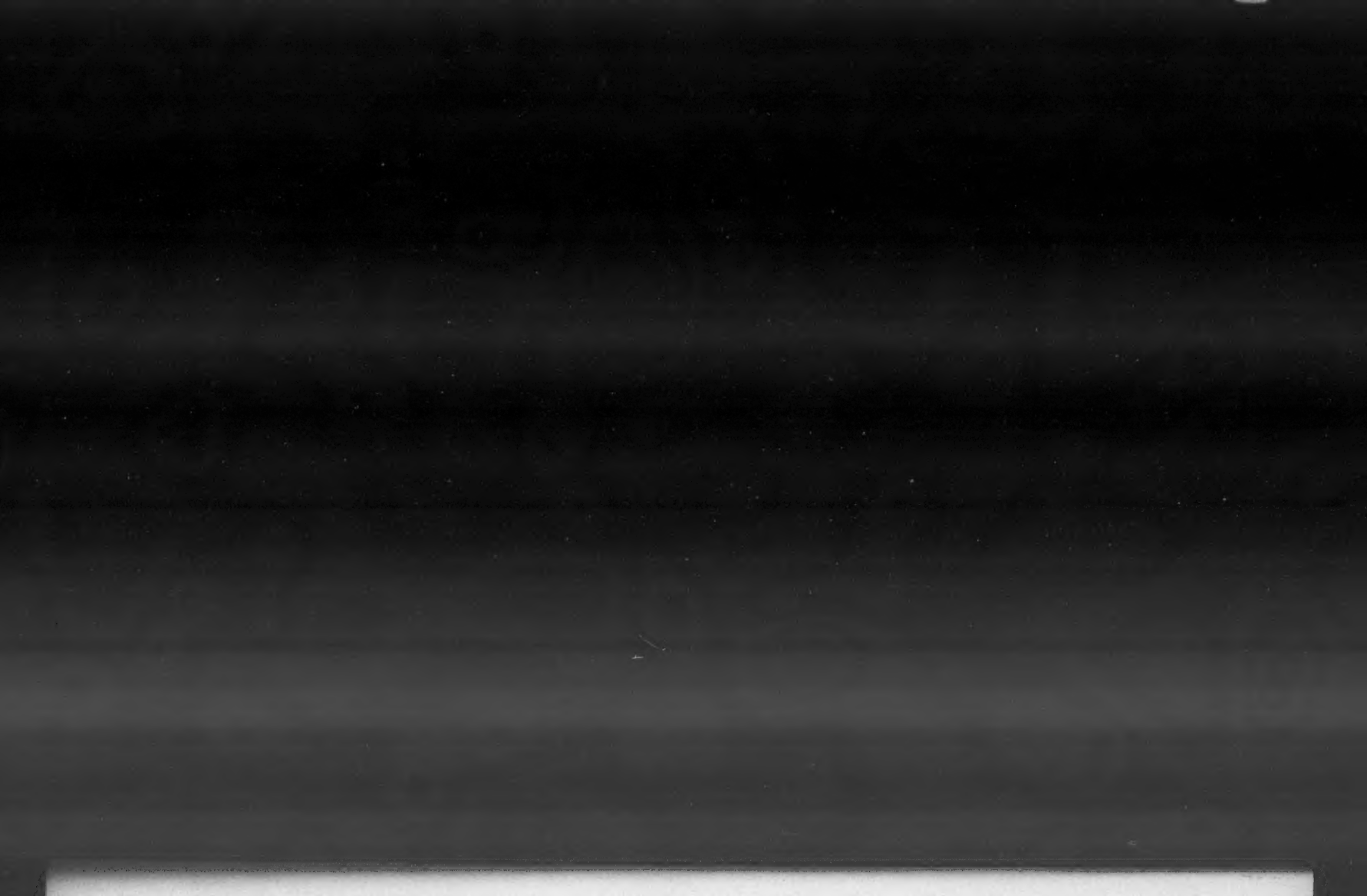
FIG. 5. Digestion of normal human skin by deoxyribonuclease at concentration of 0.01 mg. per ml. for 2 minutes, followed by direct stain with conjugate DLE I-F. Note failure to inhibit reaction. × 100.

FIG. 6. Normal immature human placenta, stained with serum from case SCL II, followed by conjugate AHG, after prior digestion by ribonuclease for 5 minutes at a concentration of 1.0 mg. per ml. Note failure of ribonuclease to inhibit positive nuclear binding reaction. × 260.

FIG. 7. Homologous positive nuclear staining of normal human kidney by conjugate SCL IIb-F. Direct technique. × 100.

FIG. 8. Abolition of nuclear binding reaction by auto-inhibition. Normal human kidney stained with serum from case SCL II, followed by conjugate SCL IIb-F from the same specimen. × 100.

FIG. 9. Failure of cross-inhibition to abolish nuclear binding. Normal human kidney stained with serum from case DLE I, followed by conjugate SCL IIb-F. Note weak staining of nuclei in glomerulus. × 100.



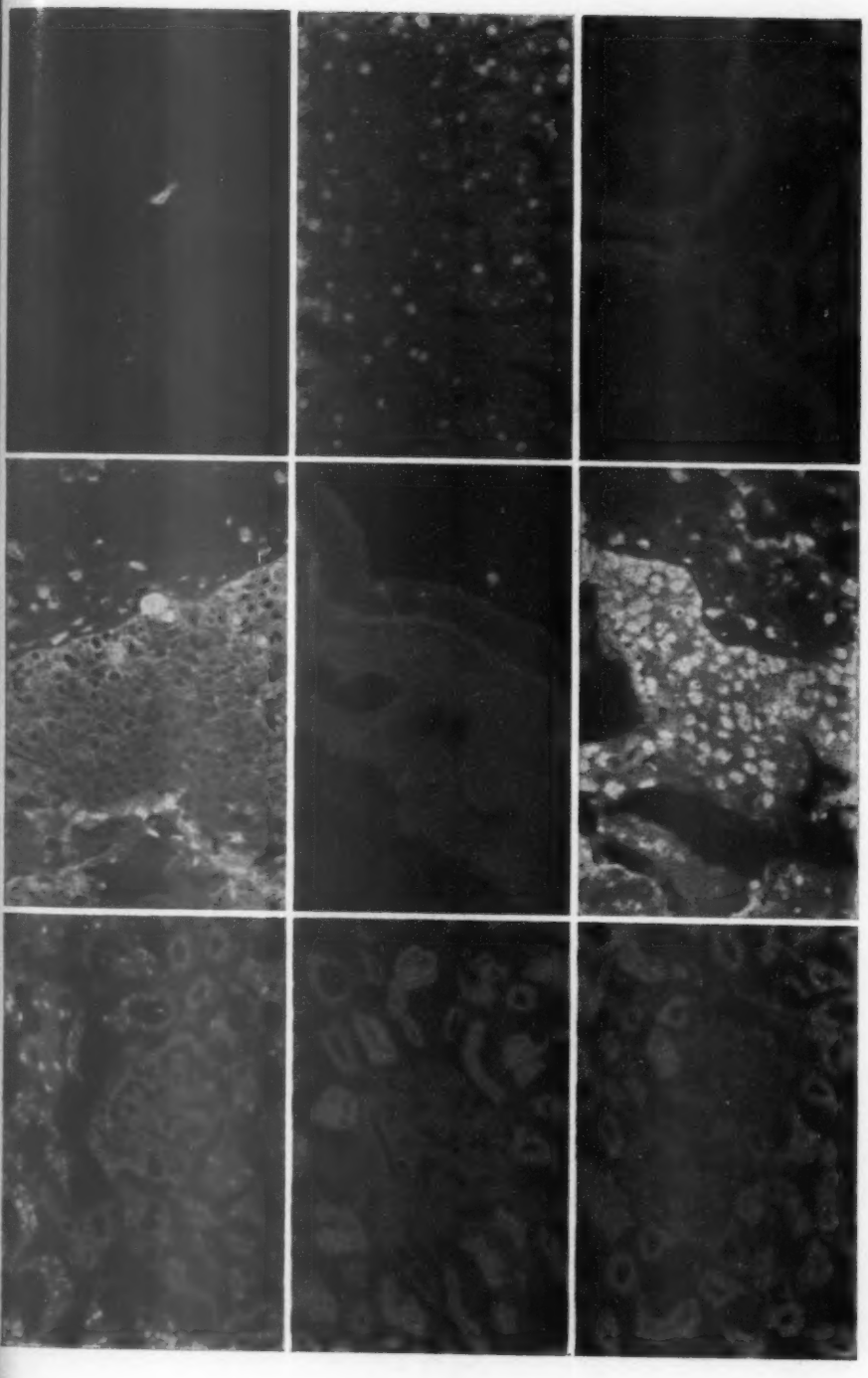


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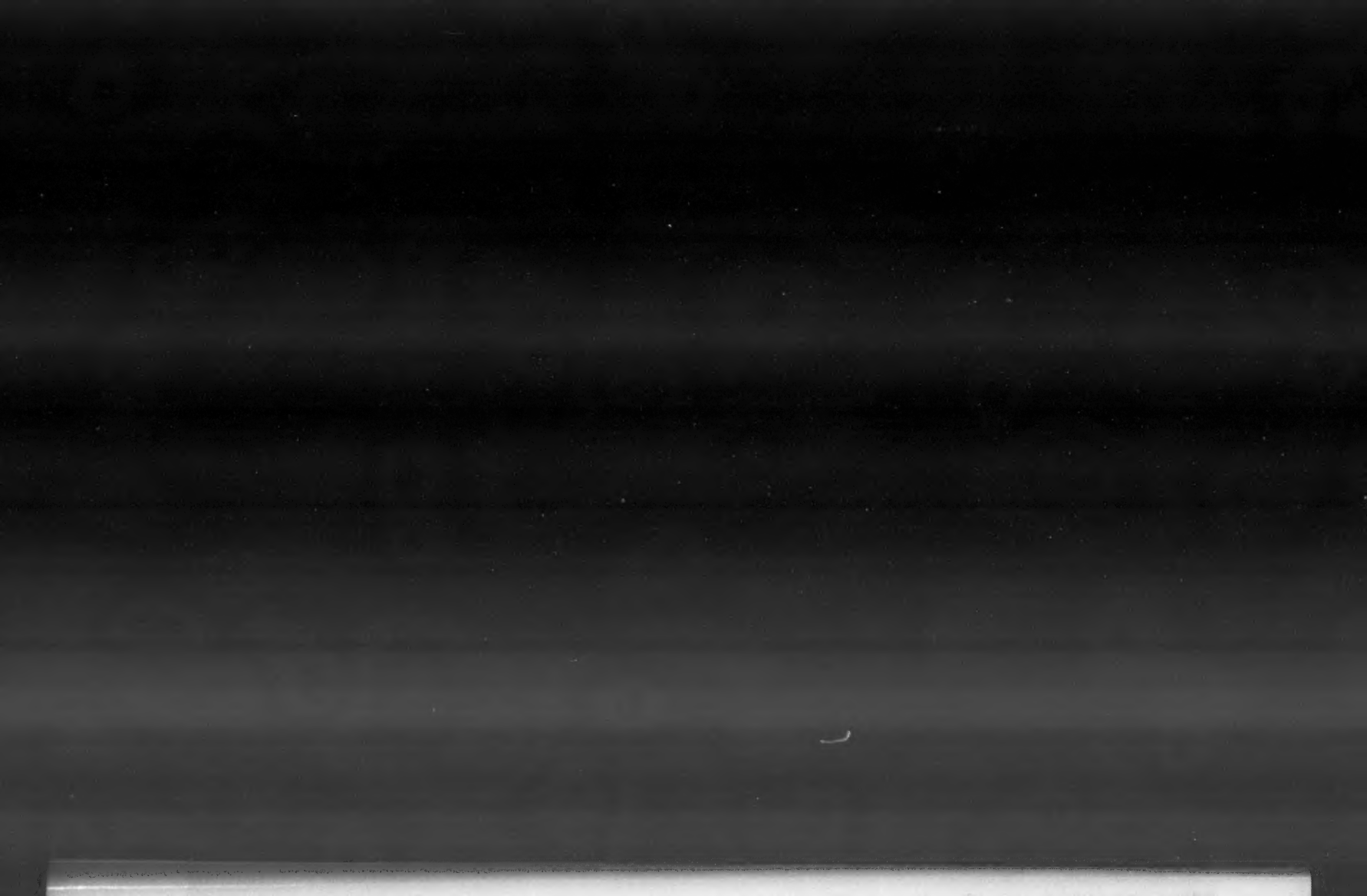
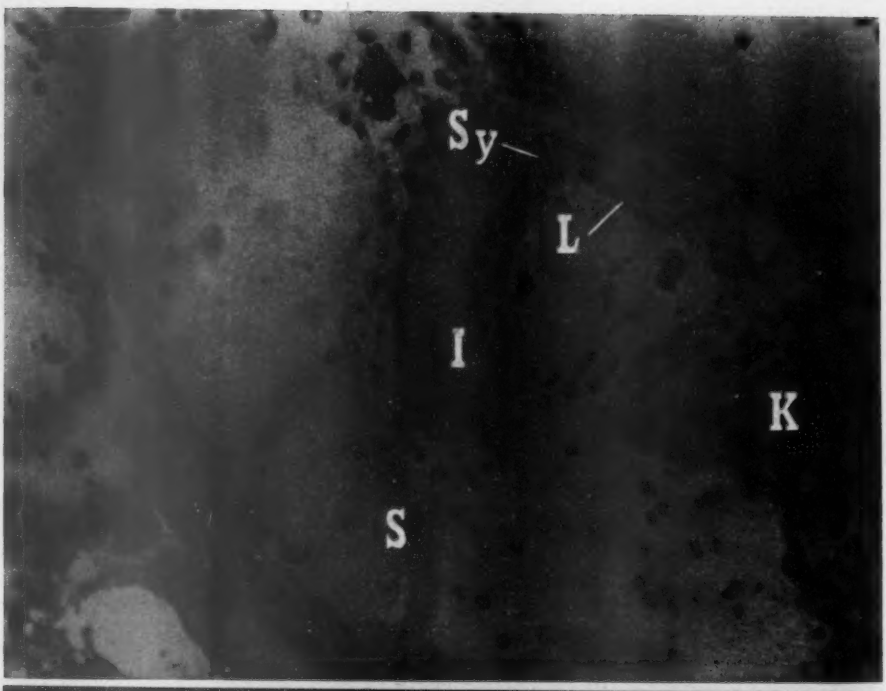
FIG. 10. Normal immature human placenta, showing general topography of a single villus in cross section. Frozen section, stained with hematoxylin and eosin and photographed by visible light. I, intervillous space; K, syncytial knot; L, Langhans layer; S, stroma in core of villus; Sy, syncytial trophoblast. × 260.

FIG. 11. Same section as shown in Figure 10, after direct staining with conjugate SCL Ia-F. Note positive nuclear binding reaction, verified by counterstain in Figure 10. Photographed by ultraviolet light. × 260.





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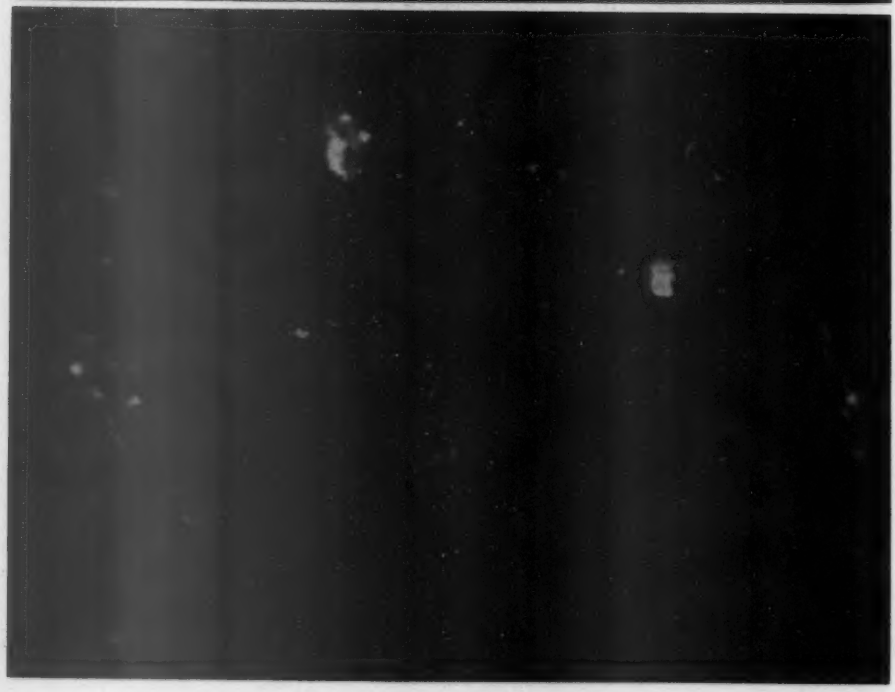

11

STUDIES ON THE BLOOD BRAIN BARRIER

I. Effects Produced by a Single Injection of Gram-negative Endotoxin on the Permeability of the Cerebral Vessels *

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The existence of a so-called "blood brain barrier" has been postulated since 1913, when Goldmann[1] demonstrated the peculiar impermeability of the central nervous system vasculature to vital dyes. He noted that trypan blue, injected intravenously, failed to stain the brain while other organs were deeply stained. It seemed apparent that the central nervous system possessed some mechanism for selective exchange of substances from the circulating blood to the nervous tissue. The anatomic location of this barrier has been difficult to establish. Walter,[2] in 1930, pointed out that the unique impermeability of the central nervous system vasculature must reside either in the vessel walls, their pial covering, or in the glial perivascular membrane. Recent studies[3,4] by electron microscopy indicate that the cytoplasmic foot processes of astrocytes are closely applied to the endothelium of capillaries of the central nervous system. This unusual relationship is currently considered to be an important factor in the blood brain barrier.

The functional integrity of the blood brain barrier is said to be maintained under conditions of anoxia, vasodilatation, and alterations in osmotic pressure and pH of the blood.[5] Certain toxic agents such as bile salts[6] or radio-opaque contrast media[7] have been demonstrated to produce a temporary alteration of the barrier so that passage of dye substances into the brain may occur.

In previous studies[8] it was shown that a single intravenous injection of gram-negative bacterial endotoxin in rabbits produced alterations in the intramural coronary arteries. These changes consisted of intimal and medial edema and vacuolization, and disruption of the endothelial lining. In other studies[9] it was shown that the administration of colloidal iron in conjunction with an intravenous injection of endotoxin resulted in the deposition of iron admixed with hyaline fibrinoid mate-

* These studies were supported by grants from the Minnesota Heart Association and the United States Public Health Service (H-2540, A-1536, and B-1688).
Received for publication, December 3, 1957.
† Part of this work was done during the tenure of a research fellowship from the Minnesota Pathological Society.
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rial in the walls of the coronary arteries. It was demonstrated also that a high molecular weight acid polymer, sodium polyanetholsulfonate (Liquoid), when given in conjunction with endotoxin, resulted in vascular lesions of the central nervous system. These lesions were characterized by the deposition of fibrinoid material in the capillaries of the choroid plexus and by the presence of thrombi in the cerebral vessels.[10]

These observations suggested that gram-negative bacterial endotoxin, if injected directly into the circulation of the central nervous system, might be associated with changes in the blood brain barrier leading to passage of colloidal materials through the capillaries and into the substance of the brain. We were also led to investigate the effects produced by the intravenous or intra-arterial administration of endotoxin upon the passage of other substances such as fluorescein into the substance of the central nervous system.

Materials and Methods

One hundred fifty-nine hybrid albino rabbits of both sexes weighing 1.0 to 1.5 kg. were used. They were fed Purina rabbit pellets and had free access to water. Of the total number, 97 were used in the studies in which trypan blue, Evans blue, or colloidal iron were given, and 62 were employed in the experiments in which fluorescein was administered. Thirty-three animals were used as controls in the experiments, and the remainder received either an intravenous or an intra-arterial injection of gram-negative endotoxin in conjunction with the administration of one or another of the above dyes.

In each instance, a single injection of 50 μg. of the lipopolysaccharide fraction of *Escherichia coli* in a volume of 1 ml. of sterile, pyrogen-free, isotonic saline solution was given. The fraction was generously supplied by Difco Laboratories, Detroit, Michigan. The intra-arterial injection of endotoxin was carried out by isolation and proximal ligation of one of the carotid arteries. Ligation was found to be necessary in order to prevent hemorrhage following administration of the toxin. The material was introduced directly into the carotid artery by means of a sterile syringe and a 20 or 23 gauge needle. Following withdrawal of the needle, the carotid artery was ligated distal to the site of administration. The intravenous injections of endotoxin were given into the marginal ear vein.

Following administration of the endotoxin, at intervals varying from 15 minutes to 12 hours, a single injection of 2 per cent trypan blue, 2 per cent Evans blue, saccharated iron oxide (Proferrin,® Merck Sharp & Dohme, containing 20 mg. available iron per ml.), or a combination of colloidal iron with one of the dyes, was given into the carotid artery

in 2 ml. amounts. Injection of these substances was made into the same artery as that used for the administration of endotoxin. Control animals were given 2 ml. of isotonic, pyrogen-free saline solution into the carotid artery which was ligated in the usual manner. This was followed by administration of the dyes at various time intervals. In each instance the carotid artery was then ligated distal to the site of the second injection. In the group of animals in which fluorescein was used, 2 ml. of a 2 per cent solution were injected into the marginal ear vein at intervals varying from 15 minutes to 24 hours after administration of toxin. Details of the time intervals and number of animals used in the various experiments are given in the text.

The animals died or were sacrificed by Nembutal® or ether anesthesia within two hours after injection of the dye. All animals given fluorescein were sacrificed 15 minutes after administration of the dye, and the brains were examined under ultraviolet light (Wood's light) for evidence of fluorescence. Necropsy examinations were performed on all animals, and the tissues were fixed in 10 per cent neutral formalin. Sections were prepared from the brain, heart, lung, liver, spleen, adrenal glands, skeletal muscle, and kidney. Staining was accomplished with hematoxylin and eosin, or eosin alone. In addition, many sections of the brains were stained by the periodic acid-Schiff method or by Perls's method for iron. In those animals which had received colloidal iron, gross evidence of staining was obtained by placing the brain in a mixture of potassium ferrocyanide and hydrochloric acid for 30 minutes.

Results

A violent reaction was observed commonly following injection of the dye substance in animals given endotoxin. This consisted of struggling, convulsive movements, and occasionally frank seizures accompanied by neck rigidity and arching of the back. The reactions were more violent in those animals whose brains subsequently showed gross evidence of staining. Marked pupillary constriction was observed almost invariably on the side of carotid injection. However, bilateral pupillary constriction was common in the animals whose brains revealed staining by the dye substance.

Staining of the skin, gums, and eyes was noted immediately following administration of the dye. The staining was limited commonly to the side of injection, the contralateral gums and skin remaining unstained. The entire head region occasionally stained diffusely, but this did not correlate well with staining of the brain. However, it was noted that extensive, diffuse staining of the brain was present only when both eyes stained equally well. A majority of the animals with diffuse

staining of the brain died within 5 to 15 minutes following administration of the dye. It was observed also that there appeared to be a markedly increased susceptibility to anesthesia, since a much lower dose of Nembutal® or ether proved to be lethal as compared with the control animals or those in which passage of dye into the brain did not occur.

In the animals receiving colloidal substances after intracarotid injection of endotoxin, the incidence of gross staining of the brain was

TABLE I

Incidence of Cerebral Lesions Following Intracarotid Arterial Administration of Endotoxin (50 μg.) and Colloid *

Interval between toxin and colloid	No. of animals	No. with gross staining of brains	Total (gross and microscopic)	Percentage stained
Controls (no toxin)	21	1	2	10%
15 minutes	4	0	1	25%
1 hour	8	1	4	50%
2 hours	12	4	6	50%
3 hours	8	4	7	87%
4 hours	20	12	13	65%
6 hours	12	7	8	66%
8 hours	8	4	5	63%
12 hours	4	0	1	25%

* Evans blue, trypan blue, colloidal iron, or combination of colloidal iron with Evans blue or trypan blue (2 ml. volume).

greater when the dyes were given 3 to 8 hours following the administration of toxin (Table I). More regular, diffuse staining was observed when the intervals between toxin and dye were 3 to 6 hours (Figs. 2, 4 and 5). At shorter or longer intervals, the staining was often spotty or irregularly distributed, although occasionally one hemisphere was diffusely stained (Fig. 3).

In the rabbits receiving fluorescein (Table II), the incidence of fluorescence was greatest when the fluorescein was administered at intervals of 8 to 12 hours after an intravenous injection of endotoxin, or 2 to 4 hours after an intracarotid injection. In these, fluorescence was noted to be of high intensity and was diffuse (Fig. 1). In other animals in which fluorescence was detected, it was usually of low intensity and was limited in distribution to scattered areas or to one hemisphere.

Microscopic examination of the brains from the rabbits which had received colloidal substances disclosed the presence of these materials within the lumens and walls of blood vessels, and within the cytoplasm of phagocytes and neurons (Figs. 6 to 12).

In animals which died or were sacrificed within 15 minutes after administration of the colloid, it was observed that the dye was localized predominantly in vessels, lying within the lumens and in the blood vessel walls. With increasing intervals of time between dye injection and death, the dyes were observed to be spread diffusely through the

TABLE II

Incidence of Fluorescence of Brain Following Administration of Endotoxin (50 μg.) and Fluorescein *

Interval and method of administration of endotoxin	No. of animals	No. with fluoresence of brain	Percentage with fluorescence
Controls			
(fluorescein alone)	12	0	0%
1 hour			
Intravenous	4	0	0%
Intracarotid	4	2	50%
2 hours			
Intravenous	4	0	0%
Intracarotid	4	2	50%
4 hours			
Intravenous	4	0	0%
Intracarotid	7	3	43%
8 hours			
Intravenous	3	3	100%
12 hours			
Intravenous	8	8	100%
18 hours			
Intravenous	7	1	14%
24 hours			
Intravenous	5	1	20%

* 2 cc. of 2 per cent solution given intravenously.

substance of the brain (Figs. 8 and 9). Many of the ganglion cells were stained, as were the microglia and astrocytes.

Within the lumens of vessels, the dye appeared as homogeneous, blue masses (Figs. 10 to 12), but when present within the vessel walls (Fig. 11) it was commonly granular. In many instances the granules appeared to lie within the cytoplasm of endothelial cells, but this was difficult to evaluate. Alternate sections stained by the periodic acid-Schiff method showed intensely staining deposits in the same sites occupied by the colloidal materials. This substance was similar to the hyaline fibrinoid material described in other papers.[9,10]

DISCUSSION

The results of the present study indicate that the permeability of the central nervous system vasculature may be altered to allow passage

of circulating colloidal materials into the brain substance by an intracarotid injection of gram-negative bacterial endotoxin. It is also shown that soluble substances such as fluorescein may pass the blood brain barrier when given intravenously in conjunction with an intravenous or an intracarotid injection of endotoxin.

The functional breakdown of the blood brain barrier may be attributed in part to the morphologic vascular alterations represented by localization of particulate dye, admixed with hyaline fibrinoid material, within the vessel walls. These vascular lesions bear a striking similarity to those of the generalized Shwartzman phenomenon.[9]

Previous studies[9] have shown that the intravenous injection of colloidal materials and endotoxin may result in the deposition of fibrinoid admixed with colloidal particles in the walls of the coronary arteries. This was similar to the deposits observed in the cerebral vessels in these studies. Examination of other tissues showed identical fibrinoid material, admixed with colloidal substances, in the coronary arteries, splenic sinusoids, and renal glomerular capillaries. These observations emphasize the morphologic similarity between the lesions observed in the central nervous system and those which occur in other organs in the generalized Shwartzman phenomenon. Likewise, the interval between injections of toxin and dye (3 to 8 hours) required for maximal staining or fluorescence of the brain, corresponds to that required in the intravenous injections of endotoxin and colloidal substances which regularly produce the generalized Shwartzman phenomenon.

It should be emphasized, however, that the development of the Shwartzman phenomenon, as evidenced grossly by bilateral renal cortical necrosis, requires pretreatment of the animal with colloidal material, followed by an intravenous injection of endotoxin.[11] In this respect, the method used in the present study differs from that used to produce the Shwartzman phenomenon. Investigations now in progress, utilizing heparin and nitrogen mustard, may provide further information regarding this point, since these substances inhibit the development of the generalized Shwartzman reaction.[12]

The mechanisms involved in the production of the alterations of the blood brain barrier are not known at present, but several factors should be considered. These include an intermediary action of adrenal gland secretions, shock, vasospasm of the cerebral vessels, and active uptake of the dye particles by certain vascular and extravascular cellular elements.

An intermediary action produced by secretions from the adrenal medulla in response to endotoxin is suggested by the results of preliminary studies in which certain sympathomimetic amines have been

shown to produce alterations in the permeability of the cerebral vessels similar to those described with endotoxin.[13] These studies suggest also that hypotension is not of primary importance, since breakdown of the blood brain barrier can be effected by the use of vasopressor amines which produce elevations in blood pressure. It is possible, however, that sudden changes in blood pressure at the arteriolar level may be important in altering capillary permeability. It is also possible that vasospasm may play a role, but this does not appear to be the primary factor in view of the relatively long interval between injections of toxin and dye required for optimal staining or fluorescence of the brain.

It has been proposed[14] that endotoxin and colloidal materials such as trypan blue, Thorotrast or colloidal iron, may have similar actions in producing a functional "blockade" of the reticuloendothelial system. Our observations of the intracellular position of dye particles that passed into the brain substance, and the presence of particulate dye within the cytoplasm of the vascular endothelial cells, suggest that endotoxin may allow passage of materials into cells which are not normally considered to have phagocytic properties.

Although the mechanisms involved in the breakdown of the blood brain barrier are poorly understood at this time, the results of the present study emphasize again the importance of gram-negative bacterial endotoxin in the production of alterations in the functional or structural integrity of the vascular system. This action of endotoxin, in increasing the permeability of the cerebral vessels, may provide more information concerning the anatomic location of the blood brain barrier. It may help to clarify the pathogenesis of many poorly understood human disorders and aid in an evaluation of the sites of action and the effects produced on the central nervous system by certain drugs.

Summary and Conclusion

Hybrid albino rabbits received a single intravenous or intracarotid arterial injection of 50 μg. of gram-negative bacterial endotoxin. At varying time intervals thereafter, one group of animals received an intracarotid injection of 2 ml. of trypan blue, Evans blue, colloidal iron, or a combination of iron and one of the blue dyes. Another group received an intravenous injection of 2 ml. of 2 per cent fluorescein.

In those receiving endotoxin and one of the colloidal substances, approximately 60 per cent showed diffuse gross staining of the brains when the interval between injections of toxin and dye was 4 hours. The incidence of breakdown of the blood brain barrier, as manifested by gross staining of the brain, decreased to 13 per cent when the

interval between toxin and dye was only 1 hour, and to 0 per cent when the interval was 12 hours. In the group receiving fluorescein, the greatest incidence of fluorescence of the brain occurred when the interval between intravenous injections of toxin and dye was 8 to 12 hours.

Microscopic examination disclosed the presence of dye particles in the lumens and walls of the vessels, and in phagocytes and neurons. The vascular lesions were characterized by the presence of subendothelial deposits of hyaline fibrinoid material, similar to that which characterizes the lesions of the generalized Shwartzman phenomenon.

The morphologic similarity of these lesions to those which occur in other organs in the Shwartzman phenomenon is discussed. Although the mechanisms concerned in alterations in the permeability of the cerebral vessels are not known, preliminary studies suggest participation by secretions from the adrenal gland.

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[*Illustrations follow*]

LEGENDS FOR FIGURES *

FIG. 1. Gross photograph of brains from 2 animals which had received a 2 ml. intravenous injection of 2 per cent solution of fluorescein. Photographed under ultraviolet light. The specimen on the right is from an animal which received a single intravenous injection of 50 μg. of endotoxin, followed in 12 hours by an intravenous injection of fluorescein. Note diffuse fluorescence of brain. The specimen on the left is from a control animal which received only fluorescein. Note absence of fluorescence.

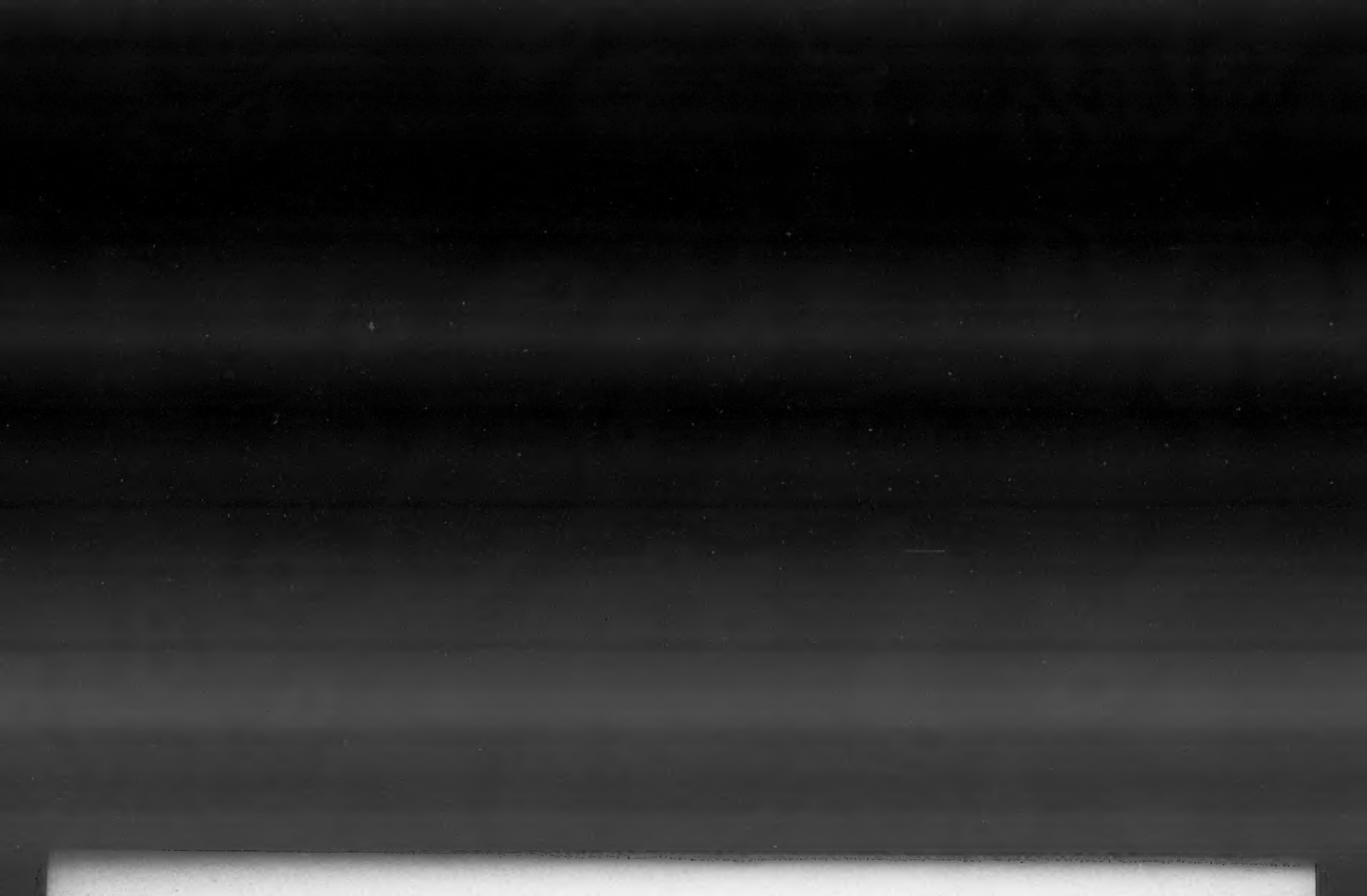
FIG. 2. Gross photograph of a brain from an animal which received an intracarotid injection of 50 μg. of endotoxin, followed in 3 hours by an intracarotid injection of 2 ml. of a mixture composed of equal parts of 2 per cent trypan blue and colloidal iron. Specimen stained in a mixture of potassium ferrocyanide and hydrochloric acid. There is diffuse, regular staining of the vessels and substance of the brain.

FIG. 3. Brain from another animal which received 50 μg. of endotoxin in the carotid artery, followed in 8 hours by an intracarotid injection of 2 ml. of a 2 per cent solution of Evans blue dye. The left cerebral hemisphere shows diffuse staining, while the remainder of the brain is unstained. Formalin fixation.

FIG. 4. Basilar surface of brain from a rabbit which received an injection of 50 μg. of endotoxin in the left carotid artery, followed in 4 hours by an intracarotid injection of 2 ml. of a mixture of equal parts of 2 per cent trypan blue and colloidal iron. Note extensive penetration of blue color into the cerebral substance. Animal sacrificed 2 hours following administration of the dye. The brain was stained by placing it in a mixture of potassium ferrocyanide and hydrochloric acid for 30 minutes.

FIG. 5. Superior surface of brain from another animal treated in a manner identical to that described in Figure 4, showing penetration of dye into the cerebral hemispheres.

* The photographs for Figures 1 to 12 were made by Mr. Henry Morris.



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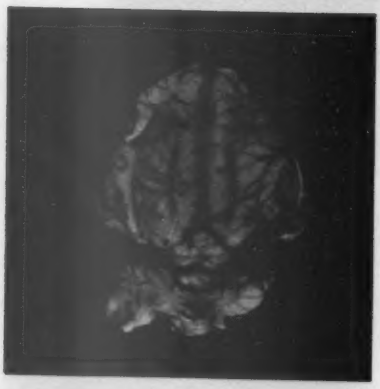

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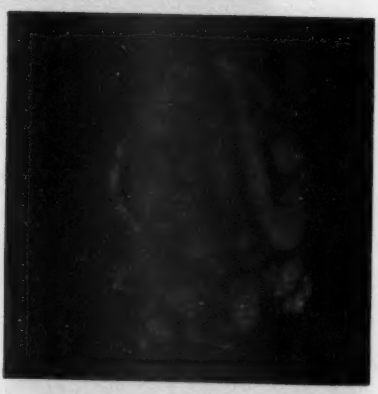

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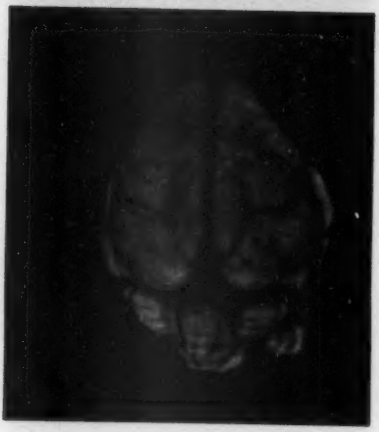

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FIG. 6. Microscopic section from the brain of a rabbit which received an intracarotid injection of 50 μg. of endotoxin, followed in 4 hours by an intracarotid injection of 2 ml. of a mixture of equal parts of colloidal iron and trypan blue. Sacrificed 2 hours after administration of the dye. Stained by Perls's method for iron, counterstained with hematoxylin and eosin. Numerous vessels containing iron-trypan blue both in their lumens and walls may be observed. Dye may also be observed in numerous phagocytic cells of the brain. × 125.

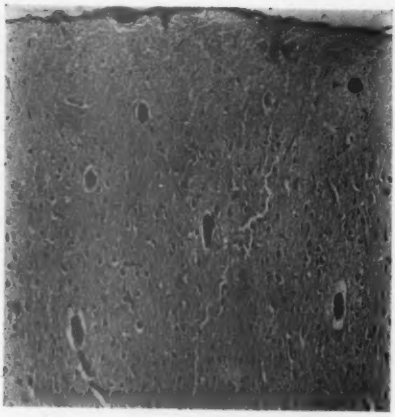
FIG. 7. Microscopic section from another animal treated in a fashion similar to that described in Figure 6. Sacrificed 15 minutes after administration of trypan blue-colloidal iron. Both the large and small vessels contain the blue dye material, but none appears to be within phagocytes. × 125.

FIGS. 8 and 9. Sections from the brains of 2 animals, each of which received an intracarotid injection of 50 μg. of endotoxin, followed in 4 hours by an intracarotid injection of 2 ml. of trypan blue. Sacrificed 2 hours following the injection of dye. Eosin stain only. Note diffuse blue background of brain. The photograph on the left shows 2 small vessels, both of which are intensely stained by the dye, and numerous phagocytic cells which contain dye. The photograph on the right is somewhat similar, but shows that numerous ganglion cells are also stained. × 125.

FIG. 10. Microscopic section from the brain of an animal which received 50 μg. of endotoxin in the left carotid artery, followed in 4 hours by an intracarotid injection of Evans blue dye. Sacrificed 15 minutes after the dye was administered. A large globule of dye is present within the lumen of the vessel. Hematoxylin and eosin stain. × 375.

FIG. 11. Section from the brain of a rabbit treated in a manner similar to that of the animal shown in Figure 10, but receiving 2 ml. of colloidal iron. Sacrificed 15 minutes after injection of the iron. Stained by Perls's method for iron. Note presence of globules of iron in the vessel lumen, and the granular particles in the wall of the vessel. × 375.

FIG. 12. Section from the brain of another animal which received an injection of 2 ml. of trypan blue in the left carotid artery 4 hours after an intracarotid injection of endotoxin. Sacrificed 15 minutes after the dye was introduced. Note presence of dye in the lumen and wall of the vessel. Eosin stain only. × 375.



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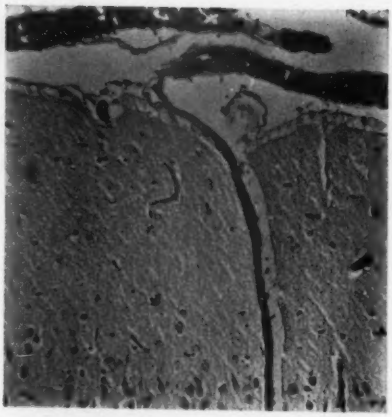

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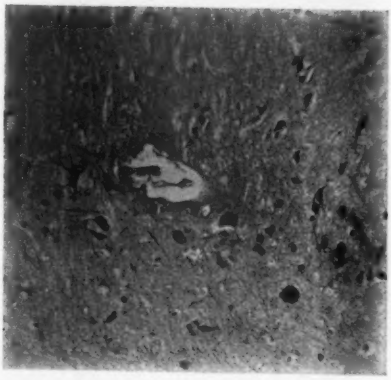

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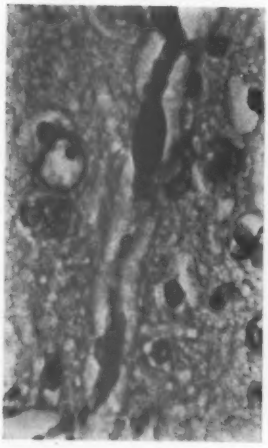

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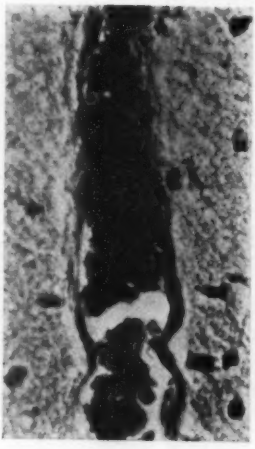

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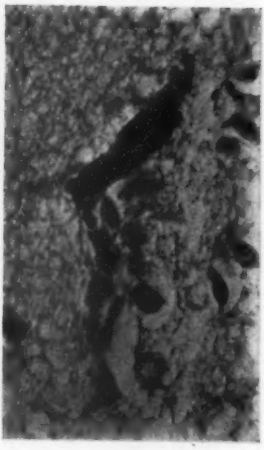

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A CLINICAL AND EXPERIMENTAL STUDY OF THE FUNCTION OF NEUTROPHILS IN THE INFLAMMATORY RESPONSE *

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Much has been written about the role of the lymphocyte in the cellular response to inflammation. The progression from lymphocyte to macrophage has been well documented by Metchinikoff,[1] Maximow,[2] Downey,[3] Bloom,[4] Kolouch,[5] and Rebuck.[6,7] The factors responsible for initiation of this cellular response were studied extensively by Menkin.[8] On the basis of his observations, he concluded that specific chemical substances were released in the inflammatory area, and one of these, leukotaxine, was responsible for attracting leukocytes to the site. He also presented evidence indicating the existence of a separate leukocytosis promoting factor. On the other hand, Moon[9] concluded that the production of leukocytosis was a nonspecific response to the introduction of protein breakdown products into the circulation. He also believed that the local events in inflammation were due to the presence of polypeptides. Polypeptides with 8 to 14 amino acids were thought to produce increased capillary permeability and leukocyte migration, while those with 5 amino acids were said to provoke leukocyte migration without increasing capillary permeability. To date, although neutrophil infiltration regularly represents an important morphologic event in acute inflammation, no one has made a systematic study of the role of the neutrophil in the inflammatory cycle. Those who have studied inflammation have noted that neutrophils are the first blood cells to appear in the inflammatory site, and their participation in the body defense has been attributed to their role in the inflammatory process. However, thus far only Rebuck[6] has postulated that the neutrophil is responsible for some of the subsequent morphologic mechanisms of the inflammatory cycle. For example, he presented evidence which he interpreted to indicate that the transformation of lymphocytes into mononuclear polyblasts and macrophages was a function of their ingestion of polymorphonuclear leukocytic debris.

One of the most striking features of the inflammatory cycle is the regular sequence of morphologic events which characterize it. Regard-

* Aided by grants from the U.S. Public Health Service, Helen Hay Whitney Foundation, American Heart Association, Minnesota Heart Association and the Minnesota Division of the American Cancer Society.
Received for publication, November 9, 1957.
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less of the nature of the irritant used to elicit inflammation, the initial event is activation of histogenous wandering cells which appear in abundance during the first hour following introduction of the irritant. Subsequently polymorphonuclear leukocytes invade the area from the peripheral blood and dominate the inflammatory process in the early hours. The exudation of neutrophils is followed in turn by infiltration with small mononuclear cells indistinguishable from the circulating lymphocytes. These cells first appear along the blood vessels and later extend throughout the inflamed tissue. If serial studies are made of the inflammatory process, the lymphocytes can be seen to become transformed into hematogenous polyblasts and macrophages. Subsequently, fibroblasts begin to proliferate and macrophages seem to be transformed into fibroblasts or resting wandering cells as the process subsides. Although the absolute time relationships may vary with the irritant used, the fundamental morphologic sequence remains constant. The constancy of the sequence of events in the acute inflammatory cycle makes it attractive to postulate that in some way each event is conditioned and induced by the one preceding it. Menkin's[10] attempt to explain the inflammatory cycle on the basis of relative resistance of the cell types to low pH, although providing a possible explanation of the sequential predominance of neutrophils, lymphocytes and macrophages during the different stages of inflammation, does not account for the sequential appearance of these cells in the exudate.

The availability of a patient with cyclic neutropenia who at times possessed no circulating neutrophils and at other times had an adequate supply of these cells provided a unique opportunity to study the validity of our hypothesis, that each morphologic event conditions and effects the subsequent inflammatory process. This patient, observed over a period of 11 years, suffered from a disease featured by regular fluctuations in the number of circulating neutrophils at 21 day intervals. During the episodes of neutropenia the circulating neutrophils fell to imperceptible levels for several days and then returned to nearly normal values. It was also established in our study of this case that during the period of neutropenia, neutrophils and their precursors were absent from the blood forming tissues as well as the peripheral blood.

In initial studies the patient received intradermal injections of typhoid-paratyphoid vaccine, Piromen®* and streptokinase-streptodornase in order that the effect of neutropenia on the development of delayed type hypersensitivity might be investigated. These stimuli were given once when the peripheral blood was devoid of neutrophils

* Piromen is a commercially available polysaccharide endotoxin derived from cultures of *Pseudomonas aeruginosa* and generously supplied for our studies by Dr. Harry Fevold of the Baxter Laboratories.

and again when the differential count revealed 42 per cent neutrophils. The patient developed erythema and induration after the administration of each of the antigens, and there was no gross difference in the appearance of the cutaneous lesions which developed during the two periods. However, punch biopsy specimens procured from the inflammatory sites 24 hours after the introduction of typhoid-paratyphoid antigen revealed a remarkable difference in the inflammatory cellularity of the exudate in the two specimens. When the patient was in neutropenic state, examination of the section showed connective tissue with only minimal perivascular round cell infiltration. Examination of the specimen taken when the patient had adequate numbers of neutrophils revealed edematous connective tissue with a profuse cellular exudate. The difference in the inflammatory process during the neutropenic and neutrophilic phases of the cycle is illustrated in Figures 1 and 2.

The interesting observation suggesting the profound effect of neutropenia on the inflammatory cycle in this patient led to attempts to study systematically the early phases of the inflammatory response in cyclic neutropenia.

Clinical Study

Method

A simple atraumatic experimental method for investigating the inflammatory cycle in man has been described by Rebuck.[1] The forearm is prepared by the application of an antiseptic. Then an area approximately 0.5 cm. square is denuded of its cornified epithelium by scraping with a sterile scalpel. Care must be taken not to cause bleeding by scraping too deeply. A drop of antigen is placed on the denuded area which is covered by a chemically clean and sterile cover slip. A small cardboard square is taped tightly over the cover slip to fix it in position. The inflammatory cells migrate onto the under surface of the cover slip and may be sampled at any time by its removal. The cover slips are replaced at frequent intervals. The exudate is fixed by quickly drying the cover slip specimens in air, and staining is accomplished with Romanowsky polychrome stains. An excellent demonstration of the progress of cellular migration and metamorphoses occurring in the inflammatory cycle may be obtained in this way.

This method was employed in the study of the patient with cyclic neutropenia. Two sites were prepared on her forearm so that 2 different antigens could be used. A drop of streptokinase-streptodornase (SK-SD) containing 100 units per cc. was applied to one site (the patient was known to be sensitive to this antigen), and a drop of 1:1,000 old tuberculin (O.T.) to which she was not sensitive was applied to the

other. The cover slips were replaced every 2 hours for the first 16 hours and at 4 hour intervals thereafter for 28 to 32 hours. At the time of the first trial the white blood cell count was 5,000 per $mm.^3$, and the differential count revealed 54 per cent lymphocytes, 35 per cent monocytes, 10 per cent eosinophils, 1 per cent basophils and no neutrophils.

Results

The response at the SK-SD stimulated site consisted of a few large mononuclear cells, probably histogenous macrophages, which appeared first at 4 hours, reached a numerical peak at 12 hours and remained until 20 hours. After 20 hours no cells were found. The response at the site stimulated with old tuberculin was similar except even fewer cells were present on the cover slip. At the end of the study the peripheral white blood cell count was 5,200 with a differential count of 17 per cent monocytes, 58 per cent lymphocytes, 24 per cent eosinophils, and fewer than 1 per cent neutrophils. Another trial was run as a check on these results 3 weeks later when the white blood cell count was 3,500 with a differential count of 1 per cent neutrophils, 69 per cent lymphocytes, 23 per cent monocytes and 11 per cent eosinophils. This time again only a few mononuclear cells were found and these only at the 12 hour point following the application of SK-SD. The other slides were completely blank. Following the application of the O.T., the 6, 8 and 10 hour samples showed a few lymphocytes, and the 12 hour specimen had a few large mononuclear cells.

The control tests were run when the patient had a white blood cell count of 3,900 with a differential count of 38 per cent neutrophils, 46 per cent lymphocytes, 6 per cent monocytes, 9 per cent eosinophils and 1 per cent basophils. The SK-SD stimulated lesion showed neutrophil migration beginning at 2 hours and reaching a peak at 4 hours. At this time lymphocytes were just beginning to migrate onto the cover slip while at 6 hours an extensive migration had taken place, and small lymphocytes constituted the predominant cell type observed. At the 10 hour stage large mononuclear cells predominated and fewer neutrophils and lymphocytes were seen. An extensive influx of neutrophils and small lymphocytes appeared again in the 12 hour sample. These were gradually replaced by large mononuclear cells which again predominated in the 20 hour specimen. In the 24, 28 and 32 hour specimens fibroblasts were present on the cover slip. The lesion to which old tuberculin had been applied showed an identical reaction for the first 10 hours. Here, however, there was no secondary influx of neutrophils and small lymphocytes at 12 hours. Large hematogenous mononuclear cells remained the predominant elements until they were

replaced by fibroblasts in samples taken during the later hours. These observations are illustrated in Figures 3 and 4. Here the inflammatory exudate which appeared during the neutropenic phase is contrasted with the inflammatory exudate occurring during the neutrophilic phase of the cycle.

Discussion

This experiment demonstrates a marked suppression of cellular migration at the inflammatory site in our patient during her neutropenic phase. The observation is compatible with the hypothesis that a lymphocytic exudation is dependent upon preliminary changes induced by an earlier polymorphonuclear leukocytic exudation. Lymphocytes, therefore, do not appear unless a polymorphonuclear leukocytic phase has taken place previously. The possibility that an antiphlogistic agent present in the patient's circulation coincidental with the neutropenia might account for this phenomenon seemed remote since studies of adrenal function revealed normal ACTH concentration, normal 17-hydroxycorticosteroid values and normal ACTH response test both when the patient's blood contained ample neutrophils and when it had none. Although the circulating blood contained approximately 10 per cent eosinophils during all 3 experiments, eosinophils were not found at the inflammatory site in significant numbers at any time. Monocytosis occurring during the neutropenic phase led to speculation as to whether or not the monocyte might assume the defensive function of the neutrophil. Our observations suggest that monocytes do not migrate into the field in the early phase of the inflammatory cycle as in the case of neutrophils, but rather infiltrate at a later time, as suggested by Rebuck.[6] During one trial in the neutropenic stage there was a rich infiltration of monocytes at 12 hours. However, during other trials only a few monocytes were found on the cover slips. This may have been due to variation in the intensity of the stimulus at the inflammatory site.

Finally, numerous studies of the inflammatory cycle were carried out in an identical manner in normal persons and in a group of patients with diseases not accompanied by neutropenia. In these instances the inflammatory process was featured by the same sequence of events as observed during the neutrophilic phase in the patient with cyclic neutropenia. We were forced to conclude that the availability of circulating neutrophils had a profound influence on the early events in the inflammatory cycle and that in their absence a markedly diminished lymphocytic invasion occurred. This, in turn, accounted for the dearth of hematogenous round cells and macrophages noted in the later stages of the inflammatory cycle.

Experimental Study

In an attempt to further evaluate the role of the neutrophil in the inflammatory response, studies were carried out upon experimental animals. An experiment was planned to investigate the time relationship of the cellular events in experimental inflammation, to study lymphocyte infiltration in normal animals and in those with neutropenia, and to determine, in addition, the effect of the local introduction of inflammatory neutrophils or aqueous homogenates of neutrophils upon the inflammatory response.

Methods

Method 1. The inflammatory cycle was studied in rabbits, utilizing the method of Kolouch.[5] In this procedure egg white labeled with carbon particles was used as an irritant. Multiple injections of the egg white were made into the subcutaneous tissue of the back, and serial surgical biopsy specimens of the connective tissue were obtained 2, 4, 8, 12, 16, 20 and 24 hours after introduction of the antigen. The connective tissue obtained by biopsy was spread in a single cell layer on a clean glass slide, fixed by rapid drying in air, and stained by the Wright-Giemsa method of Downey. By using this technique, all phases of the inflammatory cycle may be studied and the participating cells readily identified.

Method 2. For this portion of the study neutropenia was induced in rabbits by a single intravenous administration of 1.2 mg. per kg. of nitrogen mustard (HN_2). To insure an adequate supply of lymphocytes, the lymphoid tissue of the intestine, liver and spleen was protected from the effects of nitrogen mustard by clamping the celiac axis and the superior and inferior mesenteric arteries just prior to and for 5 minutes after the administration of HN_2. This method takes advantage of the fact that HN_2 is quickly hydrolyzed or fixed in the tissues following injection and is distributed by the circulation for only a very short period of time (less than 5 minutes).[11] By this means neutropenia became manifest 72 hours after the treatment and continued for 24 to 36 hours. In contradistinction, the lymphocyte count remained at normal or near normal levels throughout. We chose for experimental study only rabbits with an absolute lymphocyte count greater than 2,000 per $mm.^3$ and with fewer than 2 per cent neutrophils. In some animals sham operation was performed by opening the abdomen and clamping the arteries; nitrogen mustard was not introduced. These, as well as normal unoperated and untreated animals, were used as controls.

Method 3. White blood cells were obtained by the intraperitoneal

introduction of 100 cc. of brain-heart infusion broth containing 5 per cent glucose into two 3-pound rabbits. Sixteen hours later 200 cc. of normal saline solution was administered intraperitoneally into each animal. One hour later this fluid was removed with a 15 gauge needle and centrifuged at 1,000 r.p.m. for 15 minutes. The cells obtained were pooled and resuspended in 8 cc. of a 10 per cent solution of egg white in normal saline. The white blood cell count in the final preparation was 70,000 per mm.^{3} with 85 per cent neutrophils and 15 per cent lymphocytes. Examination in a wet chamber indicated that approximately 40 per cent of the cells were motile. Four cubic centimeters of this suspension was thoroughly homogenated in a Virtis homogenizer in order to destroy all of the cells; the remaining 4 cc. was used as an injection source of living cells.

In this experiment 3 groups of rabbits were utilized. *Group A:* Three rabbits with HN_2 induced neutropenia received egg white alone at 8 subcutaneous sites, and 3 sham-operated control rabbits were similarly treated with egg white alone at 8 subcutaneous sites. *Group B:* Two animals with neutropenia and 2 sham-operated control rabbits were injected with white cell homogenate and egg white. *Group C:* Two animals with neutropenia and 2 sham-operated control rabbits were given viable white blood cells and egg white. In addition 4 unoperated control rabbits were similarly treated. Since no differences were observed between these rabbits and the sham-operated controls, this group will not be further considered.

In each instance the inoculum marked with carbon particles was introduced into 8 sites in the subcutaneous tissues. Biopsy specimens were taken at 2, 4, 8, 12, 16, 20 and 24 hours.

Results

It will be seen in Table I that in these experiments the absence of neutrophils from the circulation (group A) interfered not only with the infiltration of neutrophils in the inflammatory sites, as would be expected, but also resulted in a marked delay in infiltration of lymphocytes as well. For example, in the control sham-operated rabbits without neutropenia in group A, lymphocytes were present in the inflammatory exudate 4 hours after injection of egg white and constituted the dominant inflammatory cell at 8 and 12 hours. To the contrary, in the rabbits with neutropenia, mobilization of the histogenous macrophages proceeded as in the controls, but neither neutrophils nor lymphocytes were present at the inflammatory site during the first 12 hours. Approximately 16 hours after the administration of the antigen in the animals with neutropenia, lymphocytes appeared in the inflam-

TABLE I

Inflammation in Normal and Neutropenic Rabbits
Effect of Leukocytes and Leukocyte Homogenates on Inflammatory Response

Time after antigen	Inflammatory response				
	Histogenous macrophages	Neutrophils	Small lymphocytes	Hematogenous macrophages	Edema
2 hrs.					
*Group A**					
Control rabbits	+	+	o	o	+
Experimental rabbits§	+	o	o	o	o
Group B†					
Control rabbits	+	+++	o	o	++
Experimental rabbits	+	o	o	o	o
Group C‡					
Control rabbits	+	++	o	o	+
Experimental rabbits	+	+	o	o	+
4 hrs.					
Group A					
Control rabbits	++	+++	+	o	+++
Experimental rabbits	++	o	o	o	o
Group B					
Control rabbits	++	+++	+	o	+++
Experimental rabbits	++	o	o	o	o
Group C					
Control rabbits	++	+++	+	o	+++
Experimental rabbits	++	+	+	o	+++
8 hrs.					
Group A					
Control rabbits	+++	++	+++	+	+++
Experimental rabbits	+++	o	o	o	o
Group B					
Control rabbits	+++	+++	++	+	+++
Experimental rabbits	+++	o	o	o	o
Group C					
Control rabbits	+++	++	++	+	+++
Experimental rabbits	+++	+	++	+	+++
12 hrs.					
Group A					
Control rabbits	+++	++	++	++	+++
Experimental rabbits	+++	o	o	o	o
Group B					
Control rabbits	+++	+++	++	++	+++
Experimental rabbits	+++	o	o	o	o
Group C					
Control rabbits	+++	++	+++	++	+++
Experimental rabbits	+++	+	+++	++	+++
16 hrs.					
Group A					
Control rabbits	+++	++	++	+++	+++
Experimental rabbits	+++	o	++	++	+++
Group B					
Control rabbits	+++	+++	+++	++	+++
Experimental rabbits	+++	o	++	++	+++
Group C					
Control rabbits	+++	++	++	+++	+++
Experimental rabbits	+++	o	++	+++	+++
20 hrs.					
Group A					
Control rabbits	+++	++	++	+++	+++
Experimental rabbits	+++	o	++	+++	+++
Group B					
Control rabbits	+++	++	++	+++	+++
Experimental rabbits	+++	o	++	+++	+++
Group C					
Control rabbits	+++	++	++	+++	+++
Experimental rabbits	+++	o	++	+++	+++

matory exudate and became the predominant cell type. Except for the absence of neutrophils, the subsequent biopsy specimens were similar to those in the normal animal (Figs. 5 to 13). The rabbits in group B which received the homogenate of white blood cells showed inflammatory responses similar to those observed in animals with neutropenia which had received injections of egg white alone. The lymphocytic exudation was again markedly delayed in its appearance as compared to the controls. One difference between the rabbits in group B and those in group A was noted. The inflammatory process appeared to be more severe in the control rabbits of group B than was the case in those of group A. It seemed clear from these observations that a homogenate of leukocytes administered to rabbits with neutropenia did not result in a restoration of the early lymphocytic infiltration regularly observed in the controls. The results of this study are illustrated in Figures 14 to 20.

The control rabbits and those with neutropenia in group C received egg white combined with viable inflammatory leukocytes. The results of this study were in striking contrast to those of the previous groups. Within 4 hours after the administration of antigen-leukocyte mixture into the subcutaneous tissue of rabbits with neutropenia, exudation of lymphocytes was present, and at the 8 and 12 hour stage (Figs. 21 to 24) lymphocytes and hematogenous polyblasts dominated the inflammatory exudate. In the group C control rabbits an inflammatory cycle similar to that observed in the control animals of groups A and B was observed. These results demonstrated that intact, viable, polymorphonuclear leukocytes at the site of inflammation were followed by the early infiltration of lymphocytes and the early appearance of hematogenous polyblasts and macrophages.

Another interesting observation made in the course of this study bears mention. As will be seen in Table I, an absence of local edema characterized the inflammatory process in the animals with neutropenia who received egg white alone or egg white combined with white cell homogenate. In contradistinction, the inflamed connective

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* *Group A:* Sham-operated normal control rabbits and rabbits with neutropenia which received subcutaneous injections of egg white only.

† *Group B:* Sham-operated normal control rabbits and rabbits with neutropenia which received subcutaneous injections of egg white plus homogenates of exudate leukocytes.

‡ *Group C:* Sham-operated normal control rabbits and rabbits with neutropenia which received injections of egg white plus viable exudate leukocytes.

§ Experimental rabbits: Neutropenia but not lymphopenia produced by the injection of HN_2; protection against the latter was effected by clamping vessels supplying intestine, mesenteric lymph nodes and spleen.

tissue of the control rabbits with normal numbers of circulating neutrophils and the rabbits with neutropenia who had received intact leukocytes was markedly edematous even within the first few hours.

Discussion

These observations appear to demonstrate that the absence of neutrophils from the circulating blood results in a dramatic inhibition of the early stages of the acute inflammatory cycle in both man and experimental animals. Not only is there a deficit in the exudation of neutrophils, as would be expected since these cells are absent from the circulating blood, but the lymphocyte (small mononuclear cell) infiltration which in the normal animal is well under way 4 hours after the administration of the inflammation provoking irritant is also significantly delayed. Indeed, in some instances lymphocytes do not appear at all at the inflammatory site in the absence of circulating neutrophils. This results in a local deficit of hematogenous macrophages in the early hours of acute inflammation. The latter cells appear to be derived from the lymphocytes or small mononuclears by a process of differentiation which begins almost as soon as these cells have gained access to the connective tissue spaces. The studies carried out in the rabbit indicate that homogenates of exudate polymorphonuclear leukocytes introduced along with the antigenic irritant are provocative of a more intensive inflammatory response in normal rabbits than occurs after injection of foreign antigenic irritant alone. On the other hand, this does not provoke the early appearance of lymphocytes (small mononuclears) in the inflammatory exudate. To the contrary, the administration of viable exudate leukocytes, mostly intact polymorphonuclear neutrophils, with the antigenic irritant, results in the prompt appearance of exudate lymphocytes which proceed rapidly in their maturation toward hematogenous macrophages. It would thus appear from these studies that whatever is contributed to the acute inflammatory cycle by the exudative polymorphonuclear leukocyte and results in the subsequent early appearance of lymphocytes and hematogenous macrophages requires viable polymorphonuclear leukocytes and not just leukocytic debris.

The key question remains; namely, how does the neutrophil in the early stages of inflammation attract lymphocytes into the loose connective tissue? The neutrophil itself might conceivably produce a specific substance or act on the connective tissue to induce production of substances chemotactic to lymphocytes. It would seem from our studies that the latter hypothesis fits better with the observations. For example, approximately 16 hours after administration of the antigen

to the rabbits, lymphocytes accumulated at the inflammatory site even in the animals with neutropenia. Perhaps in this circumstance the histogenous macrophages accumulating at the site of injection of the irritant caused an alteration in the tissue, resulting in a release of products necessary for the attraction of lymphocytes. However, the histogenous macrophages seem to produce these tissue changes at a slower rate than is the case when the early inflammation is dominated by polymorphonuclear leukocytes.

The absence of edema in the early stages of inflammation leads one to consider the relationship of cellular infiltration to the capillary permeability in inflammation. Most authors[8,9] consider altered capillary permeability as the initial feature of inflammation. Although no data are presented here which conclusively contradict this observation, these experiments seem to suggest that the sequence begins with the attraction of actively motile neutrophils to the site of irritation. Since little edema is present at the time of the earliest infiltration by these cells, it seems possible that the initial cells, at least, gain access to the injured site through capillaries that are relatively intact. It is proposed that these cells then act on the connective tissue, possibly through an enzymatic mechanism, to produce substances which increase capillary permeability. It could well be that an alteration in the capillary integrity which would permit the occurrence of local edema might be the mechanism by which the lymphocytes (less actively mobile than the polymorphonuclear leukocytes) gain access to the inflammatory area and participate in the defense reaction. The sudden simultaneous appearance of both edema of the connective tissue and lymphocyte exudation in the rabbits with neutropenia 16 hours after the introduction of antigen (Table I) lends support to this hypothesis.

Summary

1. In an extensive study of inflammation in a patient with cyclic neutropenia and in rabbits with experimentally induced neutropenia we have shown that the absence of neutrophils from the circulation profoundly alters the early events of the inflammatory cycle.

2. The early neutrophil infiltration does not occur since none of these cells are present in the peripheral blood.

3. Even when a normal number of lymphocytes are present in the circulation, the early lymphocytic infiltration and development of hematogenous macrophages from them either is markedly delayed or fails to occur entirely when the inflammatory process takes place in the absence of circulating neutrophils.

4. The development of local edema of the connective tissues which,

in the normal inflammatory cycle, is temporally related to infiltration with lymphocytes, fails to take place when neutrophils are absent from the circulation.

5. These observations are interpreted as evidence that the sequence and time relationship of events in acute inflammation in normal man and animals is a function, in part at least, of the circulating neutrophils. They also support the hypothesis that each step in the morphologic sequence in inflammation is dependent upon the preceding event.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Photomicrographs comparing the intensity of the inflammatory response in a patient with cyclic neutropenia during phases of neutropenia and neutrophilia.

FIG. 1. Biopsy made 24 hours following the intradermal injection of typhoid-paratyphoid antigen, when the patient had normal numbers of circulating neutrophils, showing dense cellular infiltration of the subcutaneous tissue. Hematoxylin and eosin stain. × 85.

FIG. 2. Biopsy made 24 hours following the intradermal injection of typhoid-paratyphoid antigen, when neutrophils were absent from the patient's blood, showing only minimal perivascular cellular infiltration. Hematoxylin and eosin stain. × 85.

FIG. 3. Sampling of the inflammatory exudate at 12 hours, obtained by Rebuck's cover slip technique, when the patient had normal numbers of circulating neutrophils, showing the abundant neutrophil and mononuclear cell exudation. Wright's and Giemsa stains. × 150.

FIG. 4. Sampling of the inflammatory exudate at 12 hours, obtained by Rebuck's cover slip technique, when neutrophils were absent from the patient's circulation, showing only a few mononuclear cells. Wright's and Giemsa stains. × 150.

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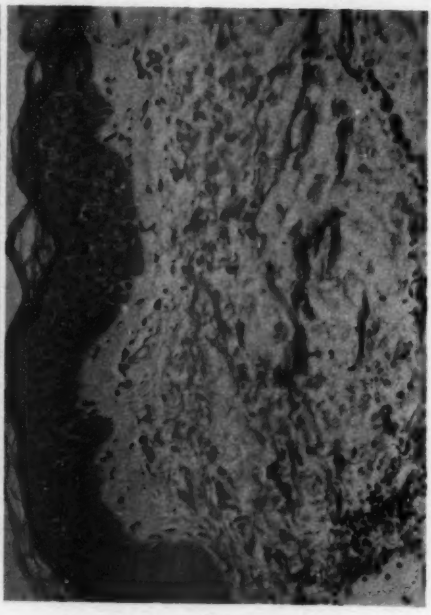

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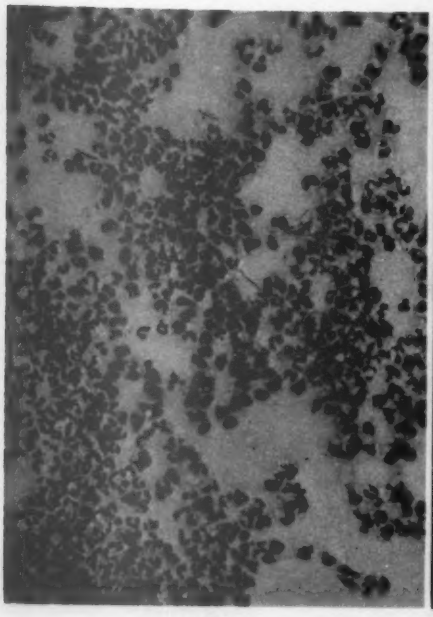

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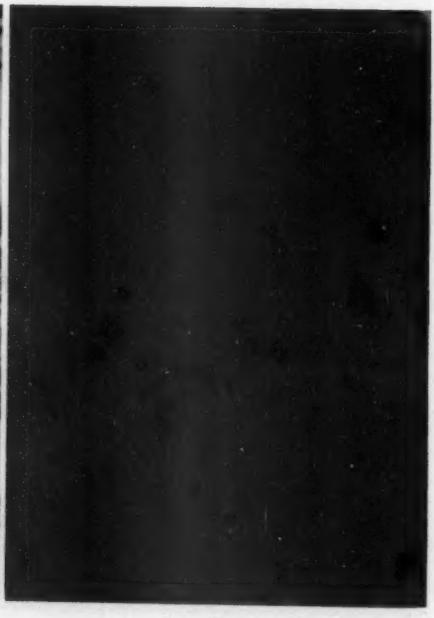

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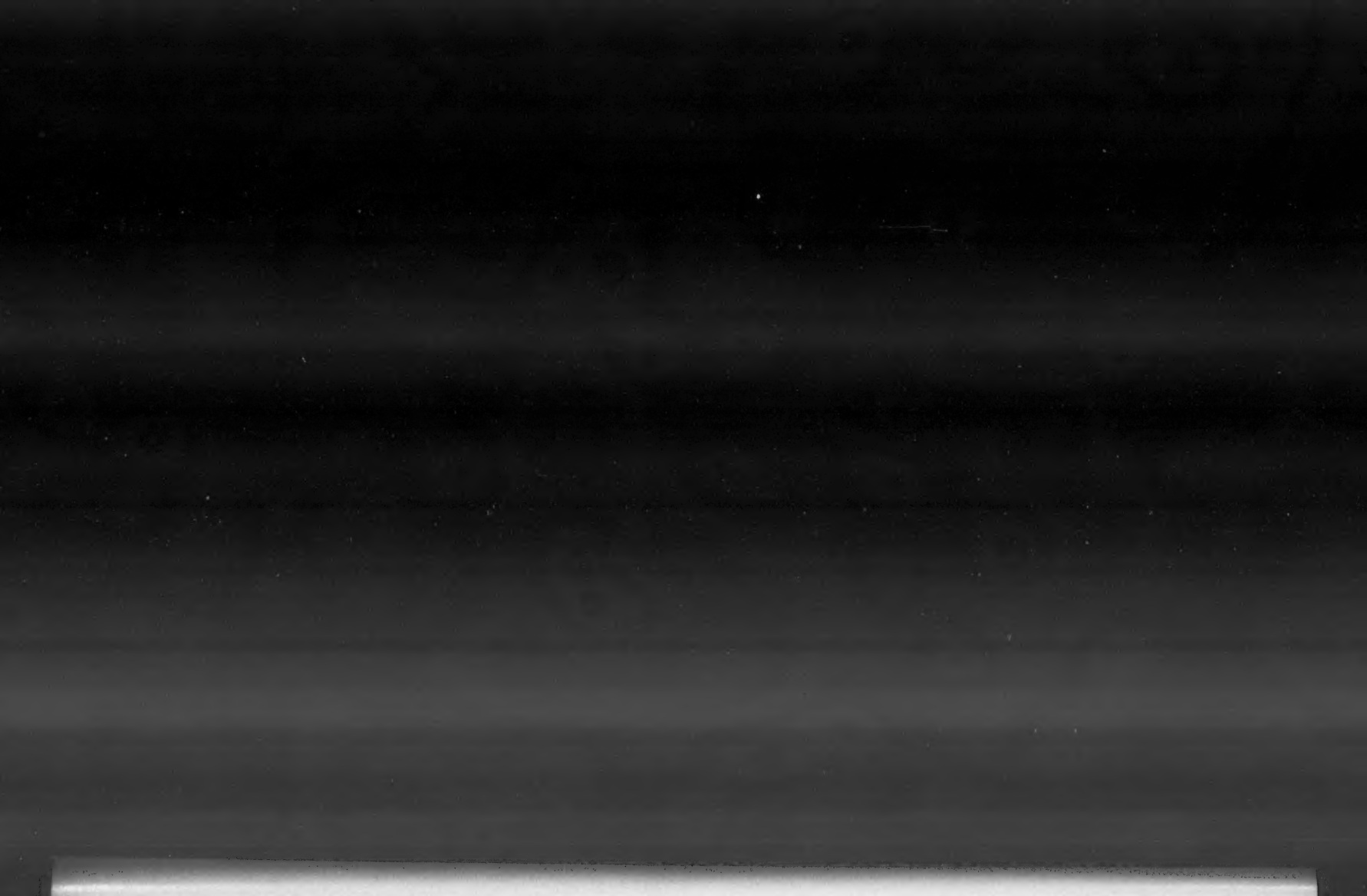
Connective tissue biopsy specimens, showing inflammation induced in normal rabbits by introduction of egg white.

FIG. 5. Inflammation at 2 hours, showing intensive neutrophil invasion. Wright's and Giemsa stains. × 150.

FIG. 6. Inflammation at 8 hours, showing lymphocyte to be the predominant cell type. Wright's and Giemsa stains. × 150.

FIG. 7. Inflammation at 12 hours, showing neutrophils, lymphocytes and macrophages. Wright's and Giemsa stains. × 150.

FIG. 8. Inflammation at 16 hours, showing intensive infiltration of lymphocytes and macrophages, accompanied by many degenerating neutrophils. Wright's and Giemsa stains. × 150.



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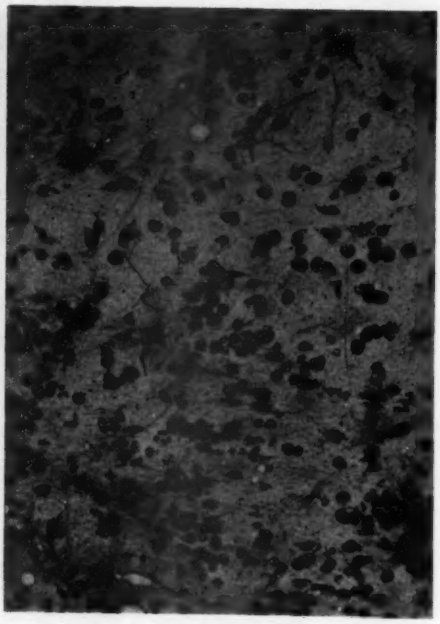

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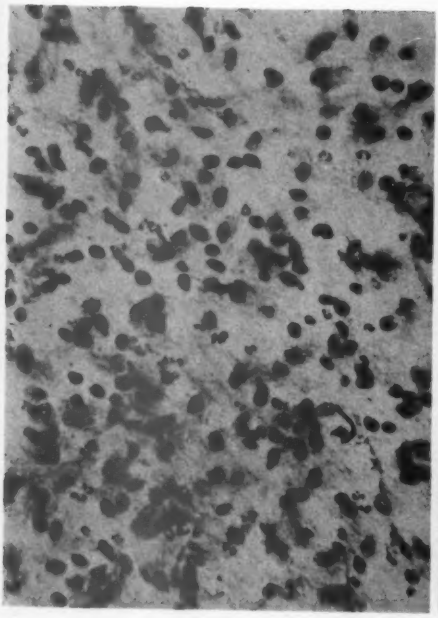

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Biopsy specimens of connective tissue with inflammation induced by egg white in rabbits with neutropenia.

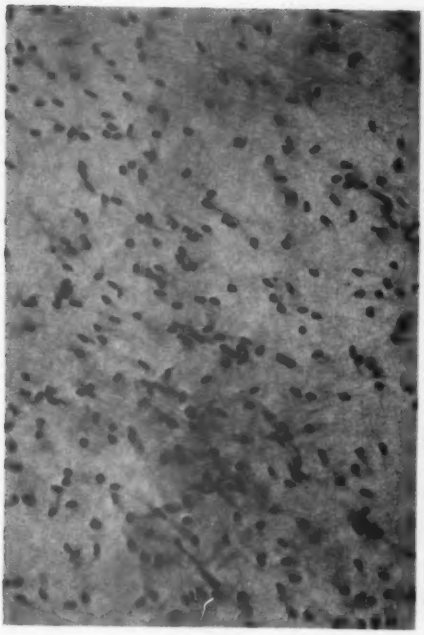
FIG. 9. Normal connective tissue prior to introduction of irritant. Wright's and Giemsa stains. × 80.

FIG. 10. Inflammation at 2 hours, showing mobilization of histogenous macrophages. Note absence of neutrophils. Wright's and Giemsa stains. × 150.

FIG. 11. Inflammation at 8 hours, showing histogenous macrophages. Note absence of both neutrophil and lymphocyte exudation. Wright's and Giemsa stains. × 150.

FIG. 12. Inflammation at 12 hours, showing histogenous macrophages. Note continued absence of neutrophils and lymphocytes from the inflammatory exudate. Wright's and Giemsa stains. × 150.

FIG. 13. Inflammation at 16 hours, showing earliest infiltration by lymphocytes. Neutrophils remain absent. Wright's and Giemsa stains. × 150.



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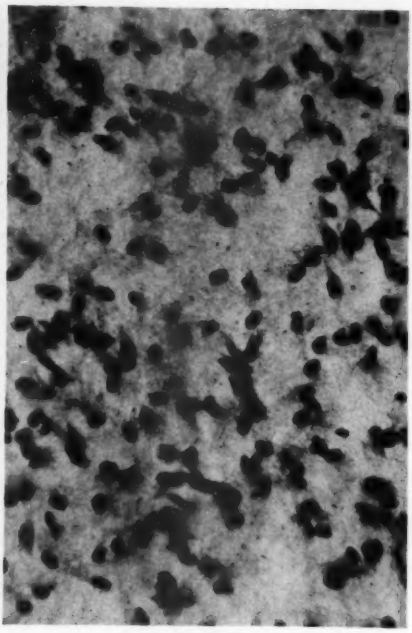

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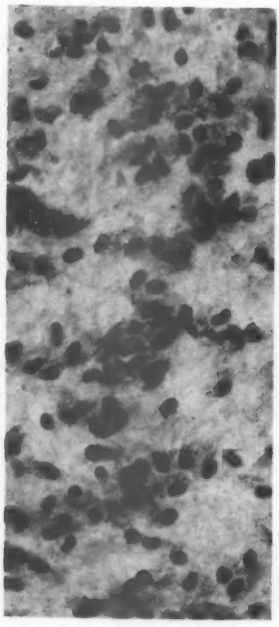

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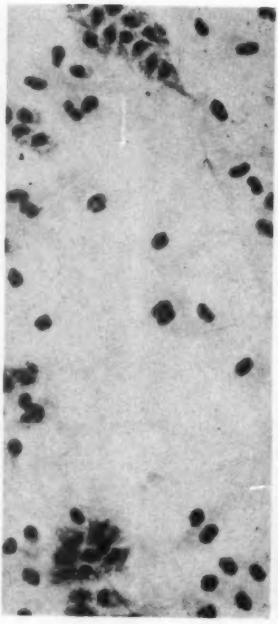

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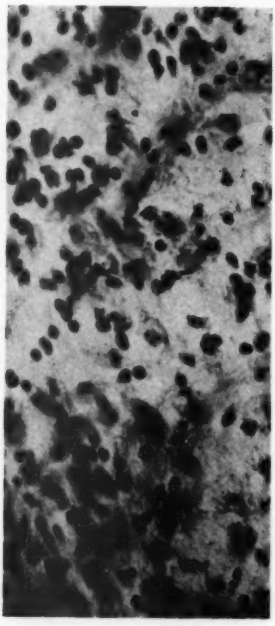

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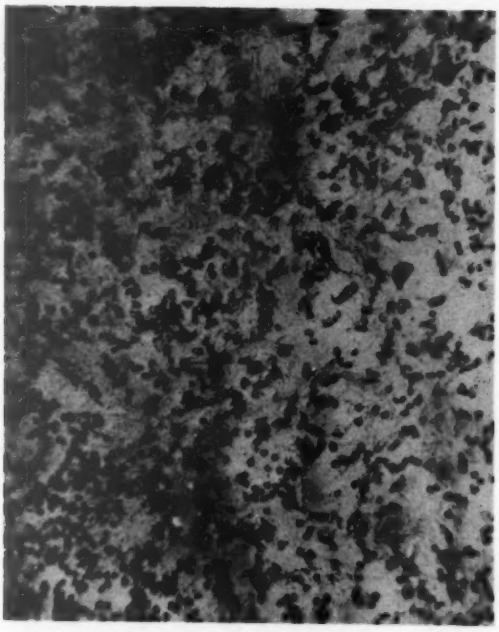
Biopsy specimens of connective tissue, demonstrating inflammation induced by homogenated leukocytes in normal rabbits.

FIG. 14. Inflammation at 4 hours, showing neutrophil and lymphocyte infiltration. Wright's and Giemsa stains. × 85.

FIG. 15. Inflammation at 12 hours, showing neutrophils, lymphocytes and hematogenous polyblasts and macrophages. Wright's and Giemsa stains. × 85.

FIG. 16. Inflammation at 16 hours, showing extensive infiltration of neutrophils, lymphocytes and hematogenous polyblasts and macrophages. Wright's and Giemsa stains. × 85.



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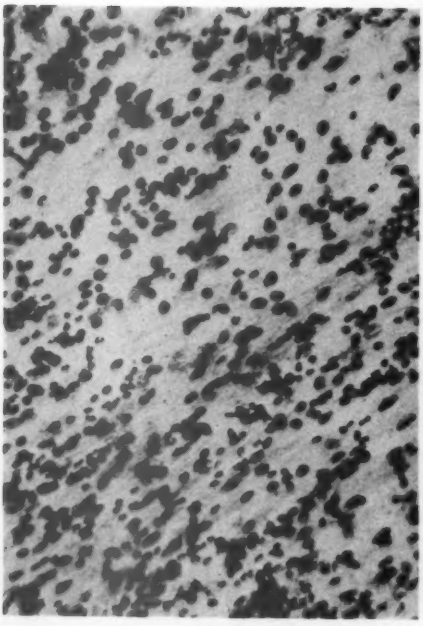

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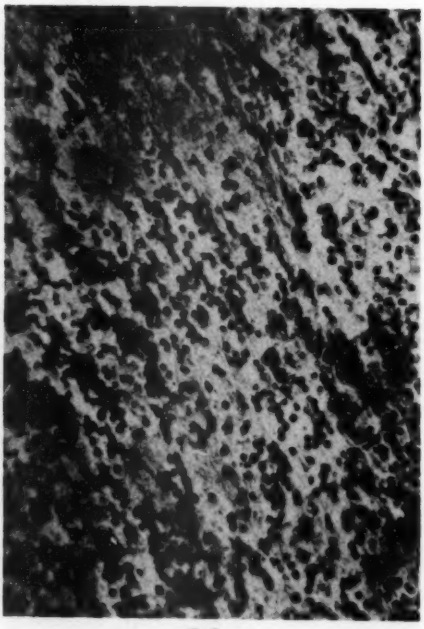

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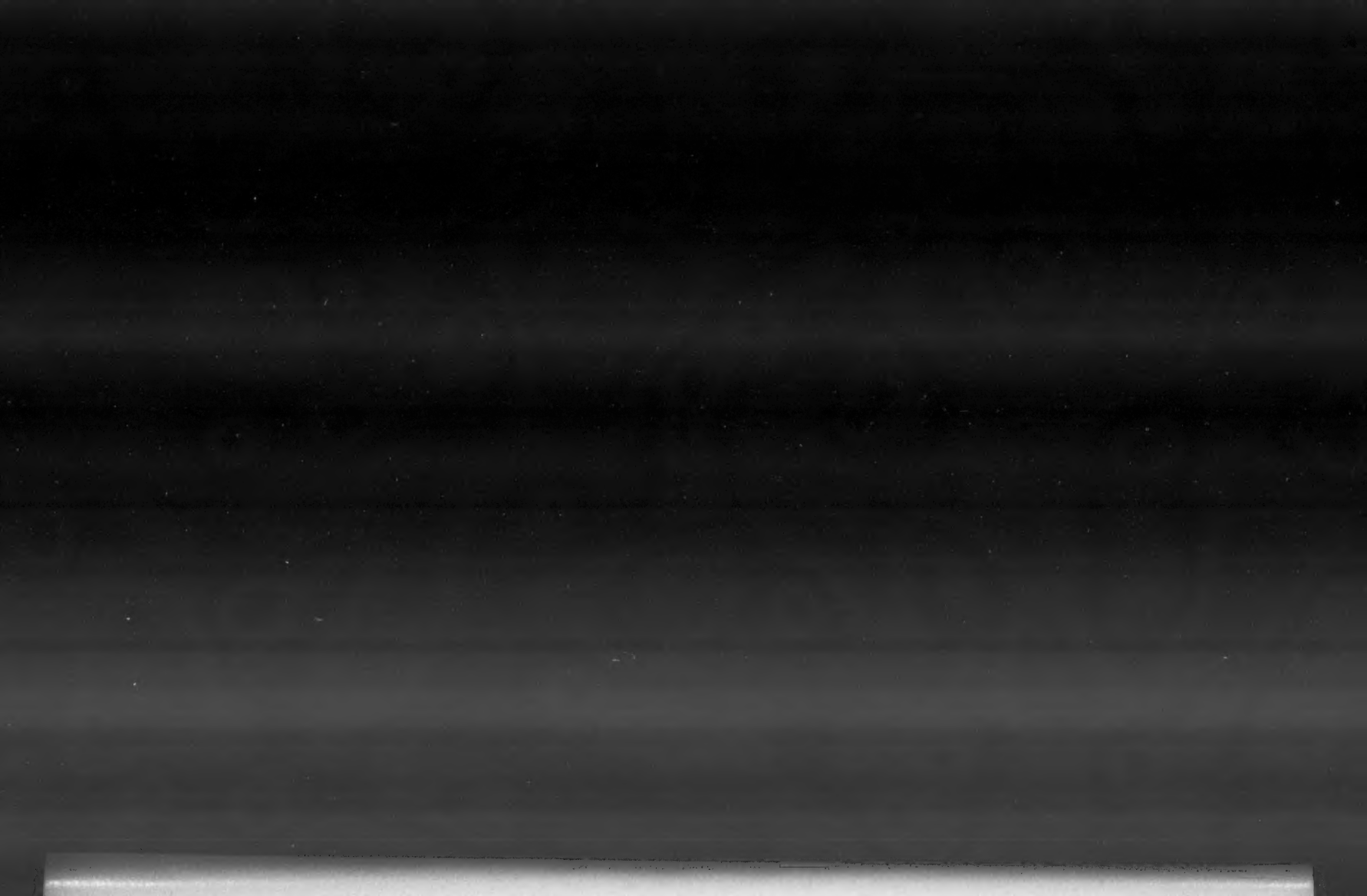
Biopsy specimens of connective tissue, demonstrating inflammation induced by homogenated leukocytes in rabbits with neutropenia.

FIG. 17. Inflammation at 4 hours, showing histogenous macrophages. Note absence of neutrophils and lymphocytes. Wright's and Giemsa stains. × 150.

FIG. 18. Inflammation at 12 hours. Note continued absence of neutrophils and lymphocytes. Wright's and Giemsa stains. × 150.

FIG. 19. Inflammation at 16 hours, showing infiltrate of lymphocytes. Note that neutrophils are still absent. Wright's and Giemsa stains. × 150.

FIG. 20. High power view of cells in Figure 19, showing hematogenous mononuclear cells. Wright's and Giemsa stains. × 800.



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Biopsy specimens of connective tissue, demonstrating inflammation induced by viable leukocytes in rabbit with neutropenia.

FIG. 21. Inflammation at 4 hours, showing histogenous macrophages and a few lymphocytes. Wright's and Giemsa stains. × 150.

FIG. 22. Inflammation at 8 hours, showing intensive lymphocyte infiltration. Note an occasional neutrophil is present. Wright's and Giemsa stains. × 150.

FIG. 23. Inflammation at 12 hours, showing lymphocytes and hematogenous polyblasts and macrophages. Wright's and Giemsa stains. × 150.

FIG. 24. High power view of cells in Figure 23, showing phagocytosis of debris by hematogenous macrophages. Wright's and Giemsa stains. × 800.

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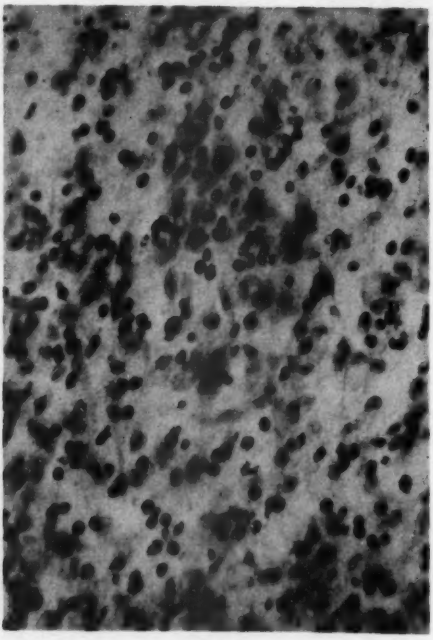

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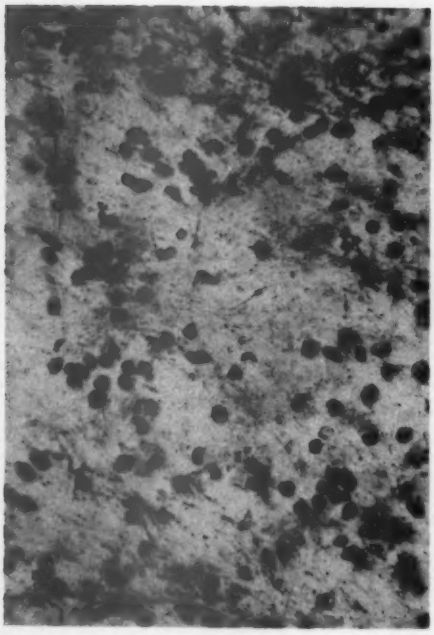

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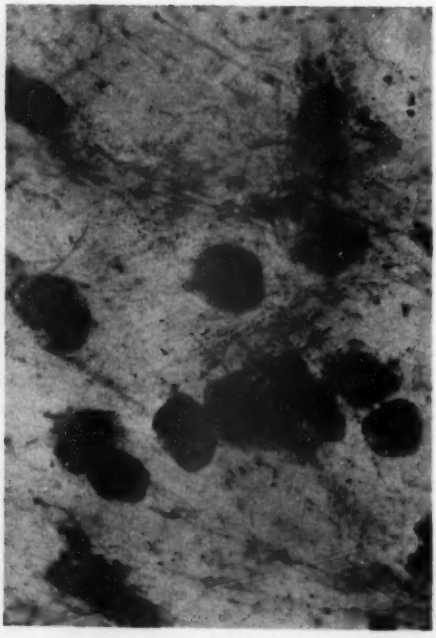

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MUCO-EPIDERMOID TUMORS OF THE BRONCHUS ARISING FROM SURFACE EPITHELIUM*

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In a study of bronchial adenomas of the carcinoid and cylindromatous types,[1] 5 tumors were encountered which closely resembled these adenomas in behavior and gross appearance, but differed in microscopic structure. This report briefly outlines the clinical and roentgenologic findings in the 5 patients bearing these tumors, with notes on the pathologic observations, treatment, and course.

The symptoms experienced by these patients were quite similar and were characteristic of slowly developing bronchial obstruction; usually, the symptoms were intermittent and often absent for long periods of time. The commonest complaints were chronic productive cough, wheezing, and hemoptysis, and the illness was punctuated by episodes of acute pneumonitis. Occasionally there were dyspnea and chest pain. No patient lost weight, and indeed, 2 patients gained considerably during the course of the disease. The longest history was 30 years, characterized by repeated episodes of hemoptysis; the shortest was 3 months, heralded by a bout of pneumonia. Three of the patients were male, 2 female. Their average age at the onset of symptoms was 45 years, the youngest being 28 and the oldest 66; at the time of diagnosis and treatment, the average age had risen to 56 years, the youngest being 43 and the oldest 66. On physical examination there were no signs except those of pneumonitis to suggest the presence of a bronchial tumor.

The roentgenologic features were of considerable importance in the determination of the presence of a lesion, its localization, and the evaluation of its resectability. In those patients in whom serial roentgen examinations were made, one of the most striking findings was the leisurely growth of the tumors. They produced gradual change in the lungs, indicating bronchial obstruction, or showed slow but relentless growth of a mass. In other words, the principal observations were those indicative of bronchial obstruction or increase in the size of a mass. The former differed in no particular way from what might be found with other lesions which cause bronchial obstruction. The slowly

* Received for publication, November 22, 1957.

growing lesion, as viewed by roentgenogram, was roughly spherical or slightly lobulated, homogeneous in density, and resembled a peripheral primary carcinoma or isolated metastatic neoplasm (Figs. 1 and 2). No lymph node involvement was demonstrated.

All patients were subjected to bronchoscopy for diagnostic purposes, and in one instance this procedure was utilized as a therapeutic measure. Although all of the lesions arose in major bronchi (3 in the right upper lobe, 1 in the right lower lobe, and 1 in the right main bronchus), 2 were not seen at the time of initial examinations. In one case this was carried out 7 and 6 years respectively before the time of ultimate resection, and in the other, repeated bronchoscopic examinations were performed. One tumor had no biopsy examination but was considered to be a bronchogenic carcinoma by the observer. In the remaining 2 patients, the tumors were visualized and biopsy specimens procured at the initial bronchoscopy. The lesion was classified as an "atypical adenoma" in one case (1935) and "consistent with adenoma" in the other (1948). At the time of later bronchoscopic examination, in 1946, of one of the patients whose tumor had not been visualized 6 years previously, a specimen was obtained which was termed "basal cell carcinoma."

The pertinent clinical data are given in Table I. The position of the tumors is illustrated in Text-figure 1.

TABLE I

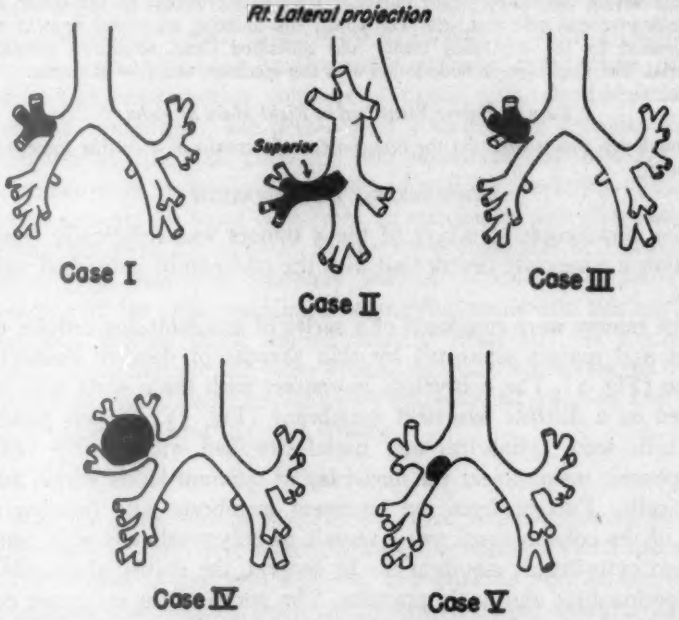
Clinical Data for Five Patients with Muco-epidermoid Tumors of the Bronchus

Case	Sex	Age at time of diagnosis	Duration of symptoms (yrs.)	Position of tumor	Treatment	Present condition	Length of follow-up (yrs.)
1	M	51	6	R. U. L.	Right pneumonectomy	Good	10
2	M	43	15	Main L. L. bronchus	Right middle and lower lobectomy	Good	8
3	M	66	3/12	R. U. L.	Right upper lobectomy	Good	6
4	F	62	30	R. U. L.	Right upper lobectomy	Good	4
5	F	59	1½	Right main bronchus	Transbronchoscopic eradication	Died during bronchoscopic resection	

Pathologic Observations

Case 1. Right Pneumonectomy

The tumor occluded the right upper lobe bronchus just proximal to its bifurcation and was attached to the mucosa at this point. Cylindrical ramifications of the tumor projected into and dilated the 3 segmental divisions of the bronchus for a distance of several centimeters. The ramifications were entirely intraluminal and were not attached to the bronchial wall. The surface of the lesion was smooth and white, its substance soft and rather friable. The bronchi beyond the tumor were ectatic; the parenchyma was collapsed and the seat of chronic pneumonitis. The middle and lower lobes were normal, and the 4 lymph nodes found in the specimen were negative grossly and microscopically.



Text-figure 1. Diagrams showing location of muco-epidermoid tumors in 5 patients.

Case 2. Right Middle and Lower Lobectomy

This 2 by 1 cm. tumor was attached over a small area to the mucosa of the main lower lobe bronchus at the origin of the superior segmental bronchus of this lobe (Fig. 3). It caused narrowing of the bronchus to the basal segments which distally showed mild ectasia. From the point of origin, the tumor projected into and caused dilatation of the superior segmental bronchus for a distance of 2.5 cm. and then branched with the bronchus, sending short cylinders of tissue into the primary divisions. The neoplasm was smooth, pale, and firm, entirely intraluminal, and attached to the mucosa only in the one area mentioned above. The projecting proximal tip of the tumor was 0.2 cm. from the resection margin of the bronchus. The middle lobe and the one lymph node included with the specimen were normal.

Case 3. Right Upper Lobectomy

The tumor was situated 1.5 cm. from the resection margin of the bronchus and formed a 1.5 cm. pale, firm mass that was attached to the mucosa at one point. Cylindrical extensions of the lesion entered and caused dilatation of the apical and posterior segmental bronchi and grew along these divisions for 1 cm.; the bronchi beyond the obstruction were dilated, and the parenchyma showed collapse and chronic pneumonitis. The neoplasm was entirely intraluminal, and there was no invasion of the bronchial wall at the point of attachment. The anterior segmental bronchus was free. The 2 lymph nodes with the specimen were essentially normal.

Case 4. Right Upper Lobectomy

The sharply circumscribed ovoid tumor measured 5.5 by 4 by 3 cm. and lay entirely within the lumen of the posterior segmental bronchus of the upper lobe. Its substance was pale and firm. The apical and anterior segmental bronchi were compressed by the expanding tumor and contained clear, tenacious, gelatinous material. The single lymph node found with the specimen was free of tumor.

Case 5. Tumor Visualized in Right Main Bronchus

This lesion was treated via the bronchoscope by means of a cutting forceps and diathermy.

Microscopic Examination

The microscopic structure of the 5 tumors was sufficiently similar to allow a composite description with the addenda of individual variations.

The tumors were composed of a series of anastomosing cellular columns and masses separated by thin sheaths of delicate connective tissue (Fig. 4). The epithelium in contact with these septa was supported on a distinct basement membrane (Fig. 5). In this position the cells were cylindrical and pseudostratified with clearly visible cytoplasmic membranes; the nuclei lay at different levels within adjacent cells. Further from the basement membrane and forming the core of the column there was a mosaic of polygonal cells with poorly defined cytoplasmic membranes. In general, the epithelial cytoplasm was eosinophilic and finely granular. The nuclei of the columnar cells were elongated and lay at right angles to the basement membrane, while nuclei of the polygonal cells were round or ovoid. The chromatin appeared as fine granules and large nucleoli were frequently present. No intercellular bridges or ciliated cells were found.

With the periodic acid-Schiff (PAS) reagent, each tumor was found to contain a number of cells with positive-staining granules or droplets (Fig. 6). All tumors showed individual cell necrosis, and in the two tumors that appeared to be the most highly organized, coagulation necrosis had occurred in multiple, sharply defined foci (Figs. 7 and 8). In these foci, measuring about 150 μ in diameter, the cytoplasm of the marginal cells was eosinophilic and the nuclei pyknotic, while the cen-

tral cells were reduced to anuclear granular masses. Often PAS-positive mucoid material had accumulated in varying quantity between the necrotic cells. Some of these areas contained a few cells with a relatively large amount of mucoid material. An extreme manifestation of this feature was the occurrence of cystic spaces containing mucoid material and lined by necrotic cells with PAS-positive cytoplasmic granules; a few of the cells were found to have desquamated into the lumens of the cysts (Fig. 9).

The description given is drawn largely from the 2 tumors that appeared to possess the highest degree of organization. The 3 other tumors adhered to the same basic pattern, but were less differentiated and consequently varied in microscopic structure. Certain characteristics remained constant qualitatively; namely, the pattern of the masses and anastomosing columns of tumor cells, the formation of basement membranes, the presence of PAS-positive granules in the cytoplasm of epithelial elements, and individual cell necrosis. The variations were probably reflections of cellular maturity. Thus, in several tumors the basal cells lying in contiguity with the basement membranes were not cylindrical but were polygonal or cuboidal in configuration and were intensely stained. Occasionally, there was crowding of the cells, associated with cytoplasmic and nuclear pleomorphism and moderate mitotic activity (Fig. 10); in the highly organized tumors, mitoses were difficult to find. Mucus production in the tumors varied from PAS-positive cytoplasmic granules and droplets to pools of mucus in cystic spaces.

Discussion

Both the gross and microscopic structure of the 5 tumors reported suggested that they were relatively benign neoplasms which had arisen in the mucosa of the bronchial tree. The growths were intrabronchial in location, and their sharply circumscribed deeper margins had not penetrated beyond the level of the cartilaginous rings. The tendency to grow along the bronchus, to expand it, and to branch with it was unusual; in several tumors a single point of attachment to the bronchial wall could be demonstrated.

In the more highly organized tumors, the microscopic appearance was distinctly reminiscent of respiratory epithelium. Pseudostratified columnar cells rested upon a basement membrane, while in the central portions of anastomosing cords of epithelium, the tumor cells became polygonal. As in respiratory epithelial elements, the tumor cells formed granules of mucoid material. Lack of a surface from which to discharge secretion or to desquamate resulted in foci of coagulation

necrosis with disintegration of the cells and release of the mucoid material. Thus, there developed minute cystlike areas filled with mucus and cellular debris. No ciliated epithelium was recognized. One fortuitous section which included the juncture of bronchial mucosa and tumor showed that the stunted ciliated cells of the pre-existing epithelium had been undermined by a layer of tumor cells (Fig. 11).

These tumors bore no microscopic resemblance to either the carcinoid or cylindromatous types of bronchial adenoma or to the bronchial adenomas arising in mucous glands as reported by Ramsey and Reimann.[2] There was a superficial resemblance of some components to epidermoid cells, but no attempt at keratinization or development of intercellular bridges was visible. In certain respects there was a resemblance to the muco-epidermoid tumors of salivary glands reported by Stewart, Foote, and Becker,[3] and by Rawson, Howard, Royster, and Horn.[4] Liebow[5] mentioned and illustrated a muco-epidermoid variant of the cylindroid form of bronchial adenoma "in which there is an intimate admixture of well differentiated mucous cells and sheets of cells suggesting squamous epithelium." In the tumors under discussion there were no well differentiated mucous glands, and intercellular bridges were not detected between the neoplastic cells. It was felt that the mucin originated in cells that stemmed from surface respiratory epithelium.

Dr. Liebow,[6] after reviewing these tumors, remarked on the necessity of further defining the interrelationships and potentialities of the group of bronchial neoplasms. These include muco-epidermoid neoplasms arising in mucous glands and surface epithelium, cylindromatous tumors, intrinsic papillomas of glandular nature, squamous papillomas, and squamous carcinomas that grow predominantly into the lumen of the bronchus.

Summary

Five intrabronchial neoplasms apparently arising from surface respiratory epithelium are described. The clinical courses of the patients, roentgenographic studies, and gross and microscopic structure of the tumors all suggest that they are relatively benign neoplasms comparable in activity to the carcinoid adenomas of the bronchus.

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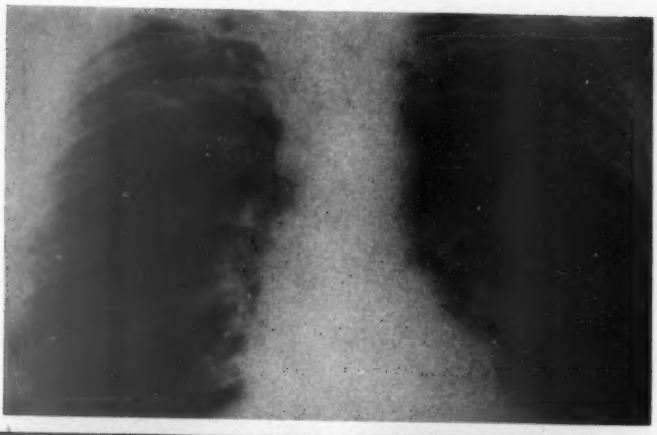
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 1. Elevation of the lesser fissure of the right lung, indicating slight collapse of the upper lobe as the only evidence of abnormality.

FIG. 2. Case 1. Six years later. Extensive changes throughout the right lung with rounded areas of increased density and rarefaction. There is considerable reduction in size of this lung as shown by the displacement of the mediastinum to the right.

FIG. 3. Case 2. The pale, homogeneous tumor expands and ramifies within the superior segmental bronchus of the right lower lobe.



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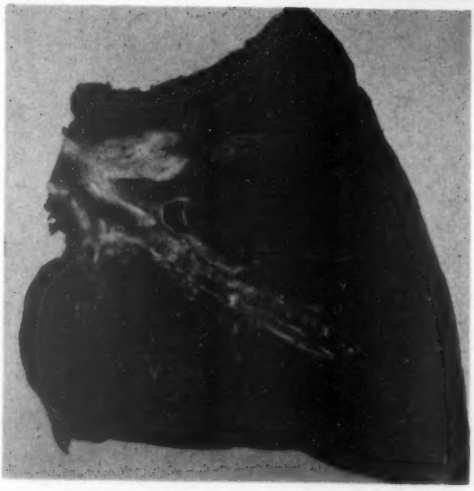

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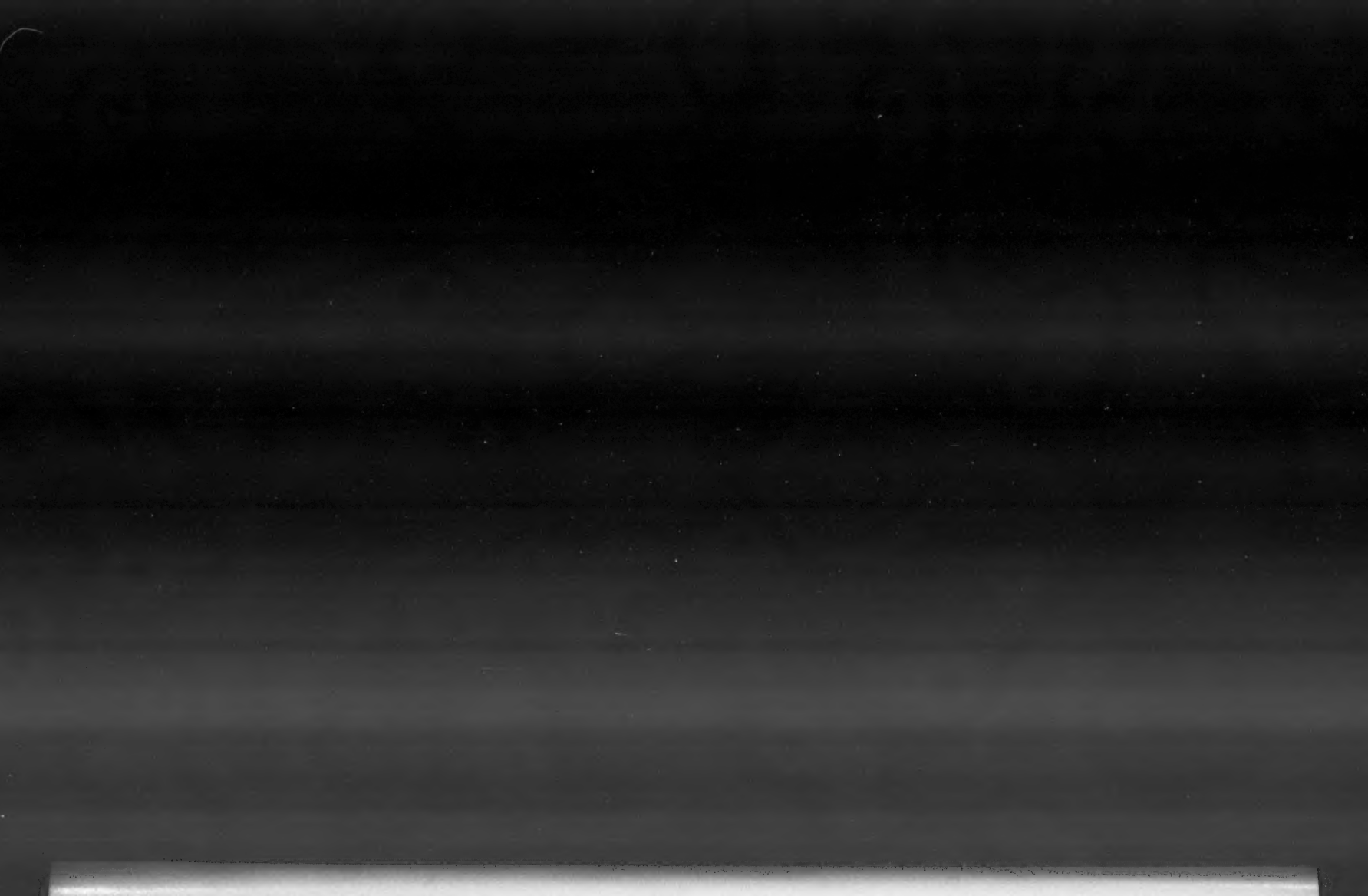
FIG. 4. Low-power view of anastomosing cords of uniform cells. Hematoxylin and eosin stain. × 125.

FIG. 5. Pseudostratified cylindrical cells supported by a distinct basement membrane. The resemblance to respiratory epithelium is quite striking. The central polygonal cells have an indistinct outline and lack polarity. Periodic acid-Schiff stain. × 500.

FIG. 6. PAS-staining granules in the cytoplasm of the tumor cells. PAS stain. × 500.

FIG. 7. Neoplastic epithelial cells before obvious necrosis has taken place. No intercellular bridges are visible. Hematoxylin and eosin stain. × 250.



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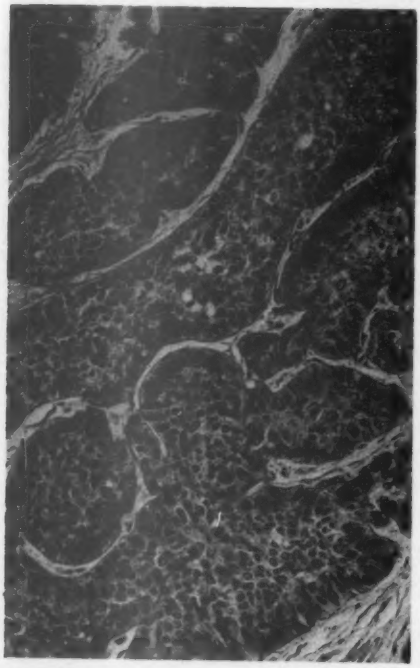

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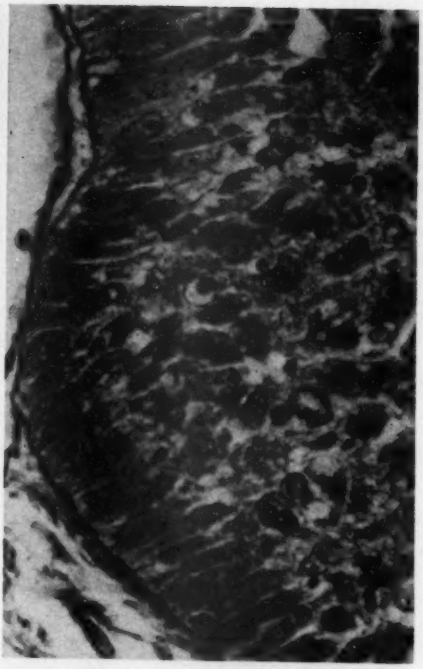

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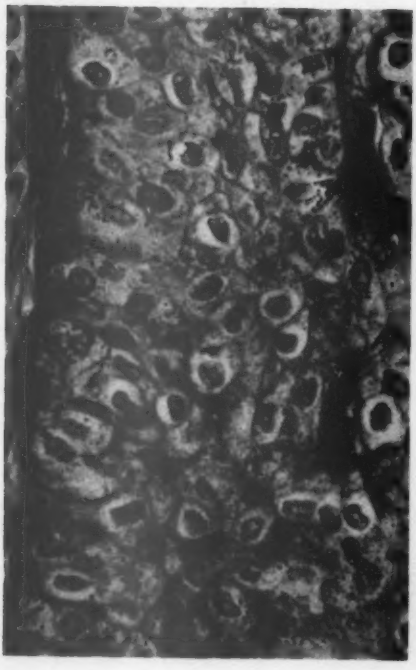

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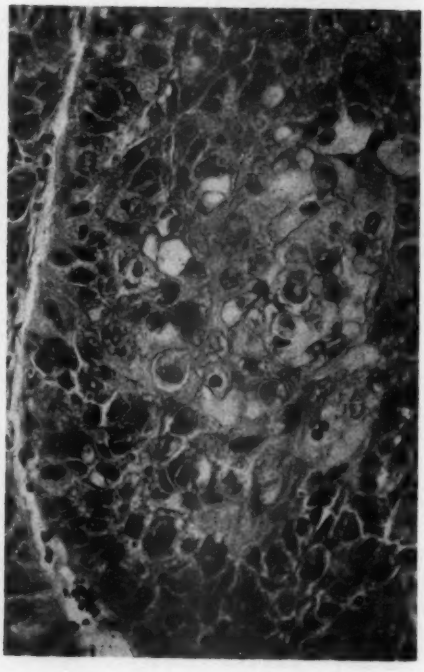

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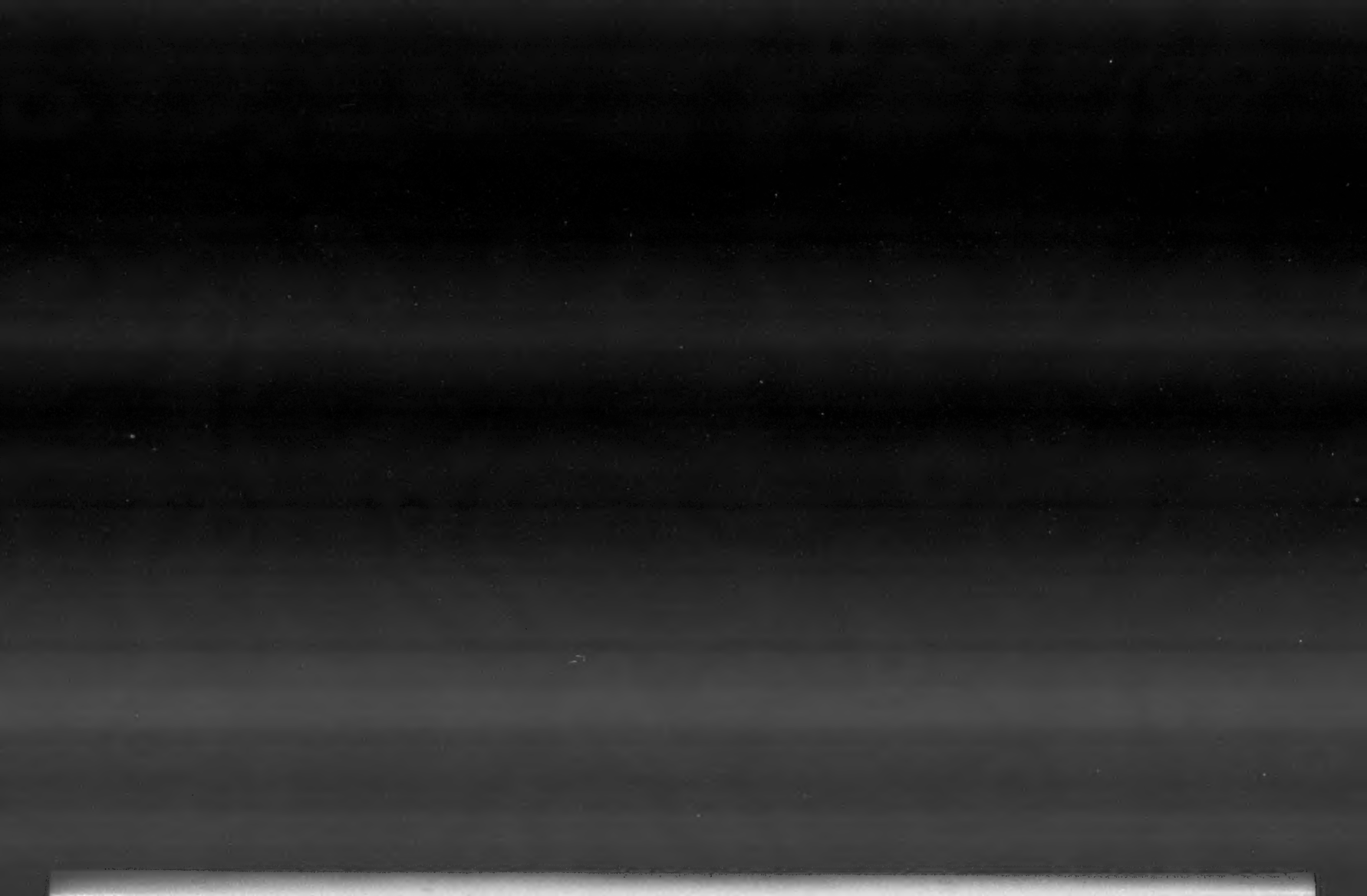
FIG. 8. Necrotic neoplastic epithelial cells. Hematoxylin and eosin stain. × 250.

FIG. 9. Cystic space containing shrunken mucoid material and a few necrotic cells. Note the cylindrical cells abutting upon the connective tissue septum. Hematoxylin and eosin stain. × 250.

FIG. 10. Loss of polarity and variation in nuclear shape and size in one of the less differentiated tumors. Hematoxylin and eosin stain. × 500.

FIG. 11. Tumor cells undermining bronchial epithelium which remains as a few stunted ciliated cells on the surface (arrows). Hematoxylin and eosin stain. × 500.



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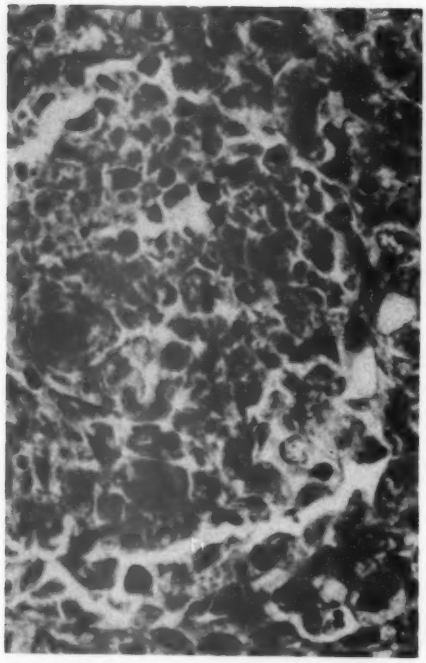

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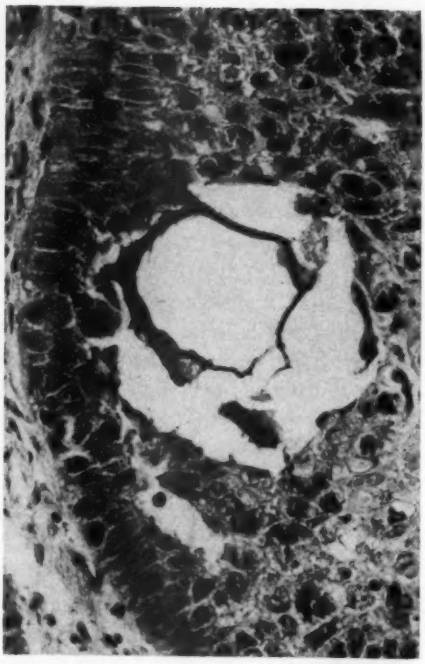

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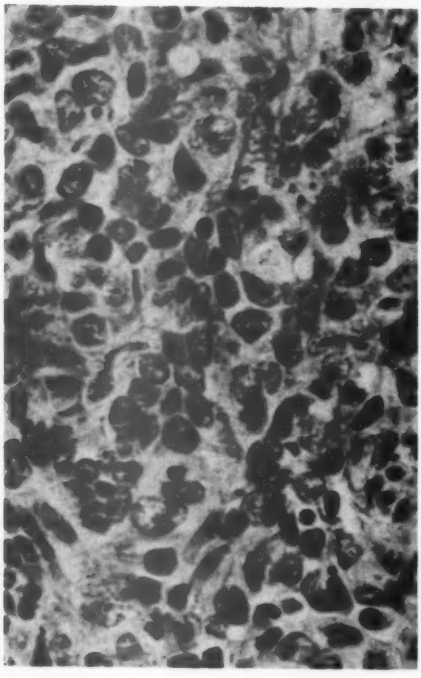

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HISTOLOGIC STUDIES OF KIDNEY BIOPSY SPECIMENS FROM PATIENTS WITH HYPERTENSION*

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In the past 20 years, since the study of Moritz and Oldt[1] concerning pathologic changes in arterioles and their relation to hypertension, the majority opinion has shifted. It was found in their necropsied cases that arterioles in hypertensive patients showed 3 chronic lesions: intimal hyalinization; medial hypertrophy and degeneration; and intimal proliferation. Taking into account clinical and experimental evidence, including that of Goldblatt,[2] Moritz and Oldt concluded that arteriolar sclerosis was more likely the cause than the effect of high blood pressure. To the contrary, necropsy studies in this period by Kimmelstiel and Wilson,[3] and Williams and Harrison[4] were thought to favor the concept of hypertension as a cause of arteriolar sclerosis. Since that time most investigators have supported the latter theory.

Ellis[5] and later Wilson[6] considered that human and animal hypertension of the Goldblatt type constituted a vicious cycle, beginning with hypertension, and through renal ischemic damage resulting in further hypertension. The reviews of renal biopsy specimens from patients with hypertension by Castleman and Smithwick[7,8] indicated that 4.2 to 7 per cent of cases had no evidence of arteriolar disease. Their material appeared to show that in some cases the hypertensive state might precede the development of renal vascular lesions; the latter could not, therefore, be considered the cause of essential hypertension. Heptinstall's renal biopsy studies[9] supported this belief, and J. P. Smith[10] from an analysis of renal arterioles at necropsy concluded that arteriolar sclerosis was aggravated by, but was not the cause of, benign essential hypertension. Findley[11] reflected that in hypertension all roads somehow returned to the kidney. He thought that a tubular enzymatic defect might be responsible for essential hypertension. In an editorial, Wakerlin[12] pointed out that in the development of human hypertension, physiologic disorders, either nervous, humoral, or both, were considered at present to be the most significant factors.

The availability of a relatively large number of kidney biopsy speci-

* Aided by grants from the American Heart Association, Massachusetts Heart Association, and the Smithwick Foundation.

Presented, in part, at the fifty-third annual meeting of the American Association of Pathologists and Bacteriologists, Cincinnati, April 27, 1956.

Received for publication, November 6, 1957.

mens removed during sympathectomy operations at the Massachusetts Memorial Hospitals in the 10-year period 1946 to 1955 has stimulated a review study of the morphologic and histochemical alterations of renal tissue removed from living persons with high blood pressure.

Surgical Materials and Methods

All microscopic sections of surgical kidney specimens filed in the Pathology Laboratory, Massachusetts Memorial Hospitals, during the 10-year period 1946 through 1955 were re-examined as unknowns. The material included 1,882 renal biopsy sections and 271 nephrectomy specimens. The histologic data recorded included descriptions of the glomeruli, juxtaglomerular apparatuses, tubules, stroma and blood vessels. Among the 30 or 40 glomeruli usually available in each section, 15 were individually assayed for hyalinization or other alterations. The tissues later found to have been removed for conditions unrelated to sympathectomy or other operations performed for hypertension were excluded from the study.

There remained available for further analysis 1,766 renal biopsy sections from 1,350 patients. In 940 cases single specimens had been removed, and in 410, renal biopsy had been bilateral. Six persons had had 3 biopsy procedures. Most operations had been performed by one of us (RHS) or his associates. A biconvex piece of kidney cortex, approximately 0.6 to 1.0 by 0.5 by 0.4 cm. was removed and fixed immediately in Zenker's solution in the operating room. During the last 18 months some specimens were divided, and portions were frozen-dried and embedded in paraffin or carbowax for special studies.

Slides already available had been stained with hematoxylin and eosin, Mallory's aniline blue, Lee-Brown connective tissue, silver reticulin, and Verhoeff's elastic tissue stains. Slides were also stained with the periodic acid-Schiff (PAS), PAS-Alcian blue and aldehyde fuchsin-PAS methods. Histochemical and ultraviolet microscopic studies are to be reported elsewhere.

A grade reflecting the degree of thickening or sclerosis of the small arterioles, chiefly those afferent to glomeruli, was established for each specimen. The grade represented the average severity of arteriolosclerosis for the specimen, an approximation, in view of the focal nature of the milder blood vessel changes. A grading of "negative" (grade 0) indicated unaltered arterioles; "grade I" represented arterioles with minor localized thickenings; "grade II," vessels, the thickened walls of which approximately equalled the lumen diameters; and "grade III" designated arterioles with wall thickness exceeding the diameter of the lumen. The presence of focal or diffuse vascular necrosis was separate-

ly noted. The classification used was comparable to that of Castleman and Smithwick,[7] except that their "grade IV" was included in the present "grade III."

When the biopsy gradings were indexed in relation to the patients from whom the specimens had been procured, some cases with specimens from both kidneys were found to have different grades of arteriolosclerosis in each. In 60 per cent of the cases the same grade was found bilaterally. The cases with dissimilar grades were tabulated in intermediate groups. To test the reproducibility of the grading method, 200 consecutive specimens were re-examined as unknowns. The grade was identical in 87 per cent of these. In 200 instances, grades assigned in the present study were compared with the original diagnoses made by other pathologists, and agreement was found in 80 per cent, indicating a reasonable degree of objectivity.

Results

For convenience, the various microscopic observations are considered and tabulated in relation to the grades of vascular alteration. It appears to be generally accepted that more severe and advanced examples of clinical hypertension are accompanied by more pronounced arteriolar nephrosclerosis.[10,12] Bearing in mind that the renal biopsy samples consisted of cortex, usually without any medulla or any medium-sized arteries, the changes found would necessarily be those that were both diffuse and located in the outer renal cortical zone. The grading for the entire group is shown in Table I.

Table I

Grades of Arteriolar Sclerosis in 1,350 Persons with Hypertension Based on Kidney Biopsy Specimens

Grades	Cases*
Negative	14
Negative–Grade I	7
Grade I	274
Grade I–II	118
Grade II	849
Grade II–III	34
Grade III†	50
Total	1,346

* Also 1 case with both negative and grade II, and 3 cases with both grade I and III arteriolar sclerosis.

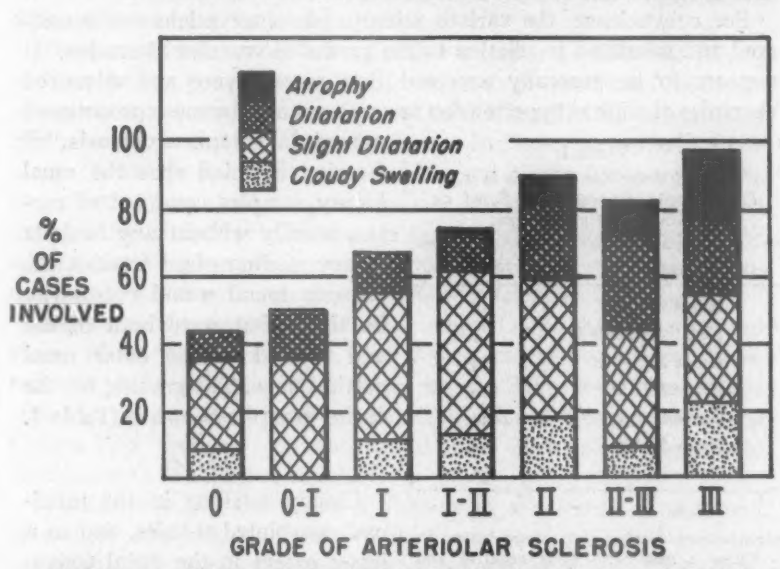
† Includes 9 cases with diffuse vascular necrosis.

Tubules

Cloudy swelling in the proximal convoluted tubules, and to a lesser extent in the distal convoluted tubules, occurred in from 7 to 22 per cent of the cases, more frequently with the higher grades of arteriolar sclerosis. The convoluted tubular epithelial cytoplasm bulged irregularly and excessively, and contained numerous, fine, distinct, or blurred basophilic granules.[7] In view of the practice of immediate fixation and the excellent histologic detail otherwise present, this

relatively minor variation was thought to be worthy of attention. It was analogous to, and could be equated with, the reversible mitochondrial fragmentation reported by Emmel[14] to occur in cells of the convoluted renal tubules of rats after 6 minutes or more of renal ischemia (Fig. 1).

Slight dilatation of the proximal convoluted tubules, characterized by both an enlargement of the lumen and a decreased height of the epithelium, was described by Shorr[15] in renal tissue procured from persons with hypertension. In the present series it occurred in over one third of the cases, with relatively equal frequency in all vascular grades (Fig. 2). Histochemical study of the tubular epithelial cytoplasm also has been reported to show an abnormally coarse staining reaction for succinic dehydrogenase, an oxidation-reduction enzyme.[15] Fajers[16] encountered a comparable microscopic lesion in the proximal



Text-figure 1. Relations between the degenerative changes of convoluted kidney tubules and the grades of arteriolar sclerosis are indicated. The percentages refer to the patients with hypertension affected, not to the number of individual biopsy specimens.

convoluted tubular epithelium of rabbits after 10 minutes of renal ischemia produced by ligature. A mild dilatation of the proximal convoluted tubules appeared to be a more extreme stage of the same general nature as cloudy swelling; the summation of these changes is shown in Text-figure 1. Strikingly dilated tubules with more rounded contours and definitely flattened epithelium, particularly of the proxi-

mal convoluted segments, were present in some of the cases. Atrophy of tubules represented further evidence of tubular retrogression.

As noted in Text-figure 1, these degenerative tubular lesions appeared in 50 per cent of the cases in the "negative-grade I group," and in 97 per cent of the grade III group. In all degrees of arteriolar sclerosis, tubular degeneration was the most frequent parenchymal lesion observed. This also constituted the earliest recognizable renal abnormality accompanying hypertension. The tubular lesions accompanying pyelonephritis were distinguished by the presence of ordinary inflammatory and reparative processes.

Interstitial Tissue

The normally inconspicuous renal stroma was infiltrated by collections of lymphocytes in approximately 20 per cent of the renal biopsy specimens obtained from "negative" and grade I groups, and in over half the specimens from the more advanced grades of vascular disease (Fig. 3). Unaccompanied as they were by plasma cells or other stigmas of inflammation, the lymphocytic foci were regarded as concomitants of renal atrophy and degeneration. In the kidney as well as in other organs, lymphocytes have been found collected at sites of basement membrane dissolution.[17] Oliver[18] and numerous other authors have emphasized the damaging effect of ischemia upon renal tubular basement membranes (Fig. 4).

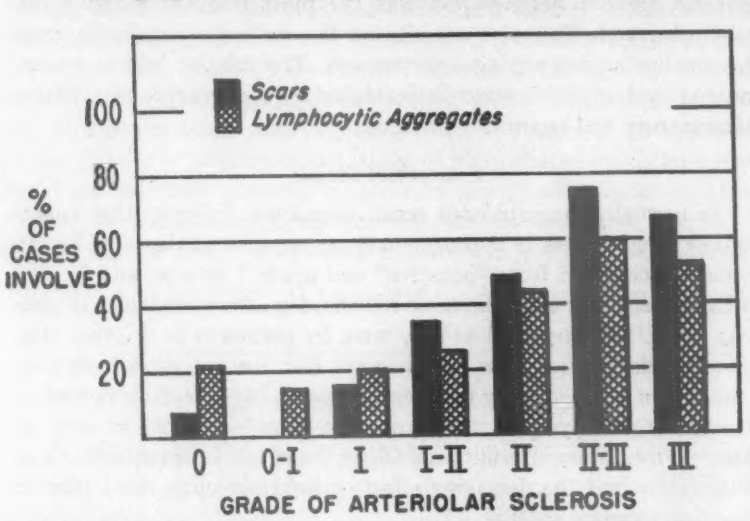
Finely pitted cortical surfaces reflect the scarring of kidneys in individuals with hypertension. Microscopically, scars with or without lymphocytic infiltration were observed in up to 74 per cent of cases with the higher grades of vascular alteration (Fig. 5). The majority of microscopic scars occurred in kidneys without other indications of inflammation; these were thought to represent reparative reactions at sites of localized atrophy and disappearance of cortical tubules[5,19] (Text-fig. 2).

Glomeruli

Most sections from the cases with less severe grades of arteriolosclerosis showed no obvious alterations of glomeruli. The earliest structural alteration identified consisted of a slight irregular stiffening of the glomerular capillary walls, due to focal thickening of the basement membrane by fibrillar acidophilic material. The glomerular loops became more obvious than normal, with an apparent simplification of the usual capillary tortuosities. Some loops sagged slightly, and in more advanced examples the entire glomerulus appeared wilted (Fig. 6). McGregor[20] and McManus[21] and others[22] have described this change in both necropsy and biopsy material.

Specimens from the group of kidneys without arteriolar sclerosis

were submitted to Dr. J. F. A. McManus for histochemical study. He found histochemical indications of glomerular ischemia even in such cases.[23] The more obvious glomerular changes have long been regarded as secondary to the establishment of arteriolar sclerosis and to



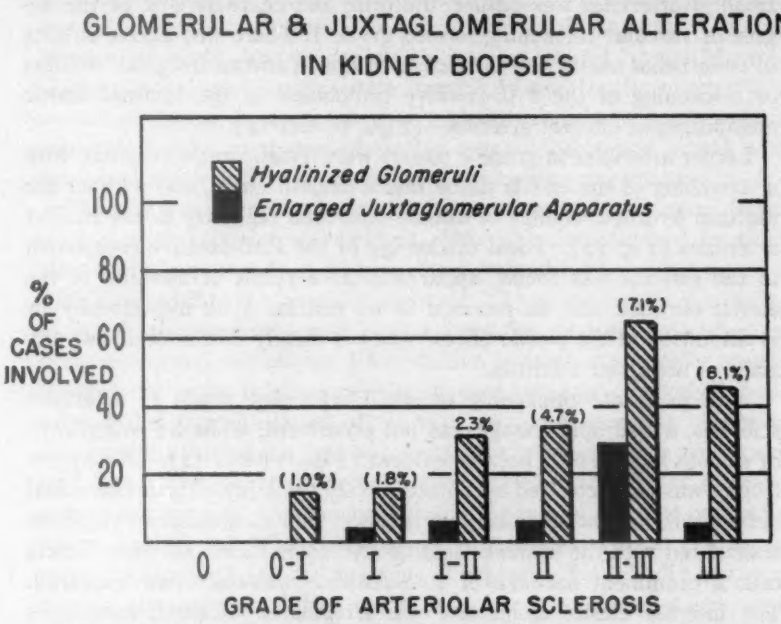
Text-figure 2. Percentages of patients with hypertension who had renal scarring or lymphocytic infiltrations in kidney biopsy specimens. Arranged according to the grades of arteriolar nephrosclerosis.

attendant mild local ischemic atrophy.[20,24,25] Hyalinized glomeruli, the end result of such regressive changes, were found more frequently in association with advanced arteriolar sclerosis. In two thirds of the patients with severe arteriolar sclerosis, there were hyalinized glomeruli, but it was estimated that only about 8 per cent of the glomeruli counted were completely hyalinized (Text-fig. 3).

Enlargement of cells with cytoplasmic vacuolization was the only alteration recognized in the juxtaglomerular apparatuses of Goormaghtigh. This occurred in from 3 to 29 per cent of cases, most commonly in the transitional grade II-III group.[26] Vacuolization was not so common in less advanced cases, and was regarded as a likely secondary manifestation of the more extreme degrees of local ischemia (Fig. 7).

Becher cells appeared as rounded groups of clear cuboidal epithelium, forming small tubules which seemed to sprout as diverticula from degenerated distal convoluted segments.[27] They were not observed except in scarred regions. These cells have been considered to

be related functionally to the juxtaglomerular apparatus. However, their presence was uncommon in the biopsy material examined. *Becher cells* occurred in 0, 0, 1.1, 2.5, 2.4, 3, and 0 per cent of cases respectively in the 7 groups of arteriolar sclerosis analyzed. The tubular buds in



Text-figure 3. The percentages of patients whose renal specimens included some completely hyalinized glomeruli and the presence of juxtaglomerular cell enlargements are shown. These are grouped according to the different grades of arteriolar sclerosis. The figures in parentheses are the percentages of completely hyalinized glomeruli in the total number of glomeruli counted.

scars were thought to represent merely localized epithelial regenerations in the distal nephrons, infrequent and of doubtful functional importance (Fig. 8).

Arterioles

Spasm of morphologically unaltered afferent arterioles, found in all but one specimen of the grade 0 group, was inferred from an abnormal concentric overlapping of the otherwise normal appearing smooth muscle cells that composed their walls (Fig. 9). Spasm was observed only once in a control series of 12 kidney biopsy specimens removed for diagnostic purposes from normotensive individuals. Artifact could not be excluded; on the other hand, spasm has not been seen commonly in the arterioles of other rapidly fixed human tissue, such as that removed for cervical biopsy.

Structural change in the afferent arterioles was first evident as a thickening of the wall due to swelling of the muscle cytoplasm; this stained very little with any method except for scattered PAS-positive granules. The hydropic alteration, or intracellular edema, of the smallest arterioles was diffuse, uniform, and characteristic of the degree of vascular thickening termed grade I. There was also a bulging of endothelial nuclei into the narrowed lumen and an irregular swelling or thickening of the PAS-positive component of the internal elastic membranes of afferent arterioles (Figs. 10 and 11).

Larger arterioles in grade I tissues were usually rather dilated, with a stretching of the elastic tissue into a smooth circle, and without the uniform hydropic change of muscle cells seen regularly in the smaller arterioles (Fig. 12). Focal thickening of the PAS-positive component of the elastica was found, apparently as a result of swelling of the elastic network and an increase in its matrix. True hypertrophy or proliferation of the elastic fibers[1] was not clearly demonstrable at any stage of arteriolar sclerosis.

With moderate thickening of small arterioles, grade II arteriolar sclerosis, a hydropic change was not prominent, while an overgrowth of smooth muscle cells became evident (Figs. 13 and 14). This hypertrophy was characterized by an excessively thick layering of individual muscle cells in concentric laminar manner, with an abundant cytoplasm stained red with the Masson-Goldner trichrome stain. Between muscle cells a prominent network of PAS-positive material often appeared. The internal elastic membrane was irregularly widened, sometimes with homogeneous nodules of PAS-positive material bulging outward. At this stage the vascular alterations in different cases, or in different microscopic fields in the same case, or along the same vessel, were much less uniform than in grade I arteriolar sclerosis. Hypertrophy of small arterioles characterized occasional specimens, while in others a swelling, nodularity and irregular PAS-staining of the internal elastic membranes and a deposition of PAS-positive material between muscle cells prevailed. In still other cases hydropic swelling, pooling of ground substance, and early indications of collagen formation were more evident in arterioles (Figs. 15 and 16).

Larger arterioles more closely mirrored the small arteriole changes in grade II sclerosis. There was an irregular but generalized thickening of the elastica by PAS-positive material, with focal nodules bulging outward. Hypertrophy of muscle cells was asymmetrical and rather irregular in location and extent. The lumens were irregularly and eccentrically narrowed.

In the grade III group the alterations became still less uniform, both

in smaller and larger arterioles. Degenerative changes predominated. The elastic tissue was no longer so clearly manifest, and, focally, a major component of the vessel wall appeared to be a pooled ground substance inundating an irregular feltwork composed of the fibrillar remnants of elastic tissue and muscle (Figs. 17 and 18). Fibrin seemed to penetrate into some vessel walls in small amounts. Collagen fibers were rarely detected in small arterioles stained by the Masson-Goldner technique. Lumens were irregularly and focally narrowed to a marked degree or were completely closed by relatively amorphous material largely composed of pooled ground substance mixed with autolyzed tissue and intercellular fluid or plasma components.

Changes were more varied in the larger arterioles. Extreme degeneration of the elastica was evident. This was characterized by thick lumps or heavy strands of an irregularly stained PAS-positive material which bulged into the lumen or outward. There was localized splitting of the elastic membrane with lacuna formation or apparent reduplication. A coarse, intercellular, PAS-positive network was usually present through the entire thickness of the affected arteriole. Endothelial and subendothelial proliferation of the type seen in endarteritis obliterans or organized thrombosis was only occasionally present in a very localized form. The large arteriolar lumens varied markedly in diameter along their courses; there were both dilatation and extreme narrowing (Figs. 19 and 20). The formation of subendothelial foam cell aggregates (atheromas) and the presence of excessive lipid[1,28] did not characterize the material studied and, in fact, were uncommon at any stage. Vascular calcification was also quite rare.

Collagen deposition in larger arterioles was found regularly in the grade III group. It was evident with the Masson-Goldner trichrome stain as a faint green network surrounded by the excessive PAS-stained ground substance. When more abundant collagen was present, it formed a layer in the midportion of the arteriolar wall, or was irregularly disposed and constituted most of the wall at some points, or made up the periphery of a vessel wall composed internally of mixed ground substance, degenerated muscle and elastic tissue. The participation of fibroblastic ingrowth was not apparent. Ultimately the collagen lost its fibrillar appearance and underwent hyaline degeneration.

Fibrinoid necrosis at any stage of arteriolar sclerosis was marked by a smudging of arteriolar wall outline and a replacement of living tissue by a fibrillar material, staining much like the fibrin in intravascular clots. The walls of affected vessels were composed chiefly of pooled ground substance, degenerated elastic tissue, and shrunken or compressed muscle cells. The fibrinoid lay in irregular intramural

masses, merging with the PAS-positive lumps and networks of pooled ground substance without regard to cell boundaries (Figs. 21 and 22). Deposition of fibrin was believed to have followed imbibition of plasma into the vessel wall, with subsequent degeneration.[29]

Venous dilatation of stellate and small interlobular venules was a regular finding in earlier stages of arteriolar sclerosis during which the efferent arterioles showed no abnormality. The explanation for the venous congestion was not evident in the cortical biopsy material, but the appearance suggested some factor encouraging splanchnic venous engorgement or possible interference with venous return deeper in the kidney. Sphincter-like activity of muscular veins at the kidney hilus appeared to be one possible explanation.[30]

Pyelonephritis

Evidences of chronic inflammation, in the form of plasma cell infiltration of interstitial tissue, dilated tubules containing colloid casts, and scars compressing atrophic tubules, were present in a minority of the specimens (13.5 per cent). Occasionally neutrophils within tubules indicated an acute exacerbation, or eosinophils mixed with other leukocytes in interstitial connective tissue pointed to a subacute inflammation accompanying chronic pyelonephritis. Healed pyelonephritis was characterized by the presence of typical dilated tubules with colloid

TABLE II

Diagnosis of Pyelonephritis in Renal Biopsy Specimens from Patients with Hypertension

Vascular grade	Percentages of cases in which pyelonephritis was observed		
	Single specimen	One of bilateral specimens	Both of bilateral specimens
Negative	14%		
Negative–Grade I		0%	0%
Grade I	5.3%	2%	2%
Grade I–II		11%	1%
Grade II	15.5%	14%	4%
Grade II–III		12%	9%
Grade III	12%	0%	0%
Totals	12.9%	11.8%	3.2%

casts simulating thyroid follicles, and scars containing atrophic tubules but without plasma cells among the interstitial lymphocytic aggregates (Figs. 23 and 24).[31,32]

As noted by Heptinstall,[9] small fragments of kidney cortex are not reliable means of recognizing pyelonephritis. In Table II are listed the percentages of cases in the various groups which were diagnosed as

pyelonephritis. Kimmelstiel and Wilson,[33] and Weiss and Parker,[34] using necropsy material, have shown that hypertension is associated with pyelonephritis in only 15 to 20 per cent of cases; these figures are comparable to the 13.5 per cent overall incidence found in the present series. Neither these necropsy studies nor the present biopsy investigations provide sufficient data to establish an accurate estimate of the real incidence of pyelonephritis in hypertension.

Unusual Observations

Unilateral renal ischemia, and a disproportionate development of arteriolar sclerosis, associated with a hypertension of the Goldblatt type, was rarely encountered. In only 2 of the 4 cases with differences of 2 grades between the degrees of arteriolar sclerosis found in bilateral renal biopsy specimens were the clinical records at least consistent with the existence of the Goldblatt phenomenon.[35] Review of the clinical records of all patients from whom negative kidney biopsy specimens were procured revealed no evidence of unilateral renal disease.[36] As a group, their hypertension was characterized clinically by a reversion to normal levels after bed rest.

A pheochromocytoma was removed at operation for hypertension in each of 13 cases during the period 1946 to 1955. Renal biopsy specimens were available from 9 cases, and in 2 there were serial biopsies. A 22-year-old man had grade I arteriolar sclerosis at the time the adrenal tumor was removed. Blood pressure preoperatively ranged between 180/115 and 240/150. There were focal thickenings of glomerular basement membranes and capillary endothelial hyperplasia; elsewhere glomerular capillaries were dilated in a focal manner. Pooled PAS-positive material of hyaline type was found in arteriolar walls and adventitia; basement membrane thickenings appeared in neighboring renal tubules (Fig. 25). Postoperatively the patient became normotensive and remained so. Six years later, at the time of cholecystectomy, a second biopsy showed that the irregular thickening of glomerular basement membranes was more pronounced. No definite arteriolar sclerosis was identified (Fig. 26). Two of 72 glomeruli counted were hyalinized. No other abnormalities were seen.

A man 21 years old had a sympathectomy, which revealed neurofibromatous malformations of the nerves and ganglia.[37] Severe hypertension persisted until almost 8 years later, when a pheochromocytoma was removed. Kidney biopsy at sympathectomy demonstrated multifocal thickenings of the glomerular basement membranes. This was associated with localized endothelial hyperplasia and dilatation of the capillaries near the glomerular roots. Arterioles exhibited grade I

sclerosis with some increase in PAS-positive material in the adventitia. The biopsy specimen taken at the time of the operation for pheochromocytoma showed grade II arteriolar nephrosclerosis, with increased amounts of PAS-positive hyalin in both arterioles and arteries. Glomeruli manifested more extensive ischemic alteration, and there were interstitial scars with compression of some tubules.

Four specimens, which on retrospective review showed some convoluted tubular vacuolization of the type since recognized as accompanying potassium depletion,[38] might possibly have resulted from primary aldosteronism. In one case a 1 cm. adrenal cortical adenoma was removed at operation. No data on potassium balance were available, but preoperatively this patient had a dilute, persistently alkaline urine which might have been due to aldosteronism. Postoperatively her condition was greatly improved. No evidence suggestive of aldosteronism was noted in the other 3 patients. Another patient, proved to have aldosteronism but encountered since this study was completed, had been treated preoperatively to restore the potassium balance. In this case no unusual renal tubular changes were found microscopically in biopsy tissue. Absence of tubular vacuolization indicative of the hypokalemic state naturally does not exclude aldosteronism; hence no conclusions can be drawn from the present material concerning the incidence of this condition in hypertensive patients.

Glomerulonephritis was found in 7 cases; these included 4 with healed lesions, 2 of the membranous type, and one with focal lesions. One case of membranous glomerulonephritis with biopsy specimens from both kidneys also showed lesions of chronic pyelonephritis with grade I arteriolar sclerosis. The other patients all had grade II arteriolar sclerosis, suggesting that two separate disease processes had affected the kidney (Fig. 27). No instance of typical chronic proliferative glomerulonephritis was encountered. Nodular intercapillary glomerulosclerosis was observed once.

Hyaline droplet nephrosis[39] occurred occasionally as an incidental finding in 10 of the cases (0.25 per cent) with bilateral biopsy specimens. Interstitial collections of large foam cells, resembling in miniature the so-called xanthogranuloma of the kidney accompanying pyelonephritis, were observed in 2 cases (Fig. 28).[40] One other section showed nodular inflammatory infiltrations resembling the lesions of interstitial nephritis which accompanies some infections or allergic diseases.

Arteriosclerosis was recognized in 4 cases from the presence of small arteries with intimal degeneration and sclerosis. Their inclusion in the specimen indicated a chance occurrence or reflected atrophy of the renal cortex.

Discussion

Less than 2 per cent of patients with hypertension were found at sympathectomy to have either insignificant or no arteriolar abnormalities in kidney biopsy specimens. Although two thirds of the cases revealed advanced renal arteriolar sclerosis, the presence of even a few individuals with apparently normal renal vessels is consistent with the view that hypertension precedes structural changes in the kidney vasculature.[7,8] The present material is probably not representative of patients in the initial stages of hypertensive disease, and there is reason to believe that the incidence of normal renal biopsy material would be higher in earlier or milder hypertension.

This finding does not, of course, exonerate the kidney from a role in the pathogenesis of essential hypertension. Under resting conditions renal blood flow may be either normal or only slightly reduced,[41,42] but it is not unlikely that the postural and emotional stresses of daily life produce significant though transient periods of ischemia in patients with hypertension. Certainly, there is adequate experimental evidence to implicate alterations in renal blood supply in the pathogenesis of hypertension.

In the present study, the existence of what appears to be spasm in otherwise normal afferent arterioles, sometimes associated with cloudy swelling of the convoluted tubules, provides evidence suggestive of the role of renal ischemia in essential hypertension. Tubular cloudy swelling and dilatation of convoluted tubules as evidences of ischemia were found antecedent to any visible alterations of arterioles. Indications of the pernicious effects of more prolonged and persistent ischemia were found in the form of lymphocytic aggregations collected about irreversibly damaged tubular segments. Ultimately, collagenization and extensive glomerular hyalinization appeared.

Vascular abnormalities were at first characteristically localized and lacked uniformity. It was inferred that the sequence of events was: spastic arteriolar contraction, intramural vascular edema, muscular overgrowth, replacement of the degenerated muscle by pooled ground substance, and, ultimately, collagenization and hyalinization.

Elastic tissue is normally probably more important in the maintenance of a steady tone in arterioles than smooth muscle.[43] Changes observed in the subendothelial elastica were thought to indicate a spreading apart of its fiber network, resulting in an increased prominence of the mucoprotein PAS-positive matrix. Degeneration and disappearance of elastic tissue were accompanied by pooling of the ground substance and its permeation of adjacent intercellular spaces. Mechanical effects of local tissue pressure may account for the localization of this PAS-

material in certain zones of arteriole walls.[17] It was not clear that any hyperplasia of elastic fibers occurred. Collagen in arteriolosclerosis was rather sluggishly formed and focal in its deposition.

Fibrinoid was found either focally or diffusely in arteriolar walls. Since it was identifiable in all grades of arteriolar sclerosis from I through III, it appeared to be a fairly commonplace event accompanying hypertension. Accelerated arteriolar degeneration appeared to account best for the presence of this substance.[44-46] No special investigation of fibrinoid was made, but an imbibition of plasma into the arteriolar wall, precipitation of fibrin and its subsequent degeneration or eventual transformation into hyalin were thought to reflect exacerbations of the elevated blood pressure. The conclusions of Gitlin, Craig and Janeway[47] from studies of fibrinoid in other diseases were believed to have likely applicability to hypertension. Experimentally induced necrosis of arteriolar muscle may also apparently result in the appearance of fibrinoid.[48,49]

Except for arteriolar spastic contraction, and the subsequent chain of degenerative changes, there was no morphologic explanation for the renal ischemia found; larger blood vessels were not available for study. It is of interest that pathologists have concluded that ischemia rather than vascular occlusion is the central factor in the production of both the cerebral[50] and the myocardial damage[51] which constitute the two other major complications of hypertension. Indications point to the afferent arterioles as the sites of narrowings which are at first functional and later structural, but other mechanisms for renal ischemia must be considered. Whether venous stasis produced by muscular sphincters near the hilus or by other means may contribute to renal ischemia is beyond the scope of this study. Anatomic structures resembling sphincters exist; these become prominent after sympathomimetic stimulation and in certain instances of unilateral renal atrophy.[30] Further attention should be paid to venous stasis in hypertension, and to the role of renal phlebitis in perpetuating or exaggerating damage to nephrons in chronic pyelonephritis.

Some of the clinicopathologic correlations derived from this series of cases are reported elsewhere.[52,53] Statistically significantly elevated diastolic blood pressures were found in association with the more advanced grades of arteriolar sclerosis. The basis of this correlation is debatable. On the one hand, it is possible that the diastolic blood pressure level contributed to the degree of vascular thickening. On the other hand, it is conceivable that secondary vascular changes might tend to intensify pre-existing hypertension further. In the kidney such vascular changes may lead to the production of a pressor substance or

substances which could then initiate a vicious circle. Whatever the nature of the relationship between renal vascular lesions and the severity of hypertension, it is obviously not a precise one, since there were wide variations in local kidney alterations at most levels of blood pressure.

Arteriolar necrosis was not found to require any specific diastolic pressure level for its development. Nor did the presence of fibrinoid, either focal or diffuse, indicate a terminal state or an inevitably poor prognosis.[54,55] In the present material it was found that no correlation could be made between diffuse vascular necrosis and postoperative survival time.[52] Fibrinoid necrosis was judged to be simply an evidence of accelerated renal damage and was reasonably reversible after sympathectomy. The clinical state of malignant hypertension and the pathologic demonstration of arteriolar fibrinoid necrosis appeared related, but not equivalent.

Summary

Kidney biopsy specimens from 1,350 patients with hypertension who underwent sympathectomy were re-examined microscopically. The earliest parenchymal alterations observed were cloudy swelling, dilatation and atrophy of some convoluted tubules, and accompanying spasm of afferent arterioles. Significant arteriolosclerotic lesions characterized over two thirds of the cases. Subsequent to spastic arteriolar contraction, intramural edema and muscular hypertrophy were thought to occur. These, in time, were followed by arteriolar elastic tissue degeneration with pooling of its ground substance and, eventually, irregular collagen deposition and hyalinization in the afferent arterioles. Scars, lymphocytic aggregates in interstitial tissue, and slight or obvious glomerular alterations commonly accompanied the more advanced grades of arteriolosclerosis. Fibrinoid necrosis was observed in arterioles in all grades of arteriolosclerosis.

It is concluded that renal ischemia probably precedes detectable vascular abnormalities in essential hypertension. When structural alterations have developed in the arteriolar walls, secondary renal changes may serve to maintain or accelerate the hypertensive process.

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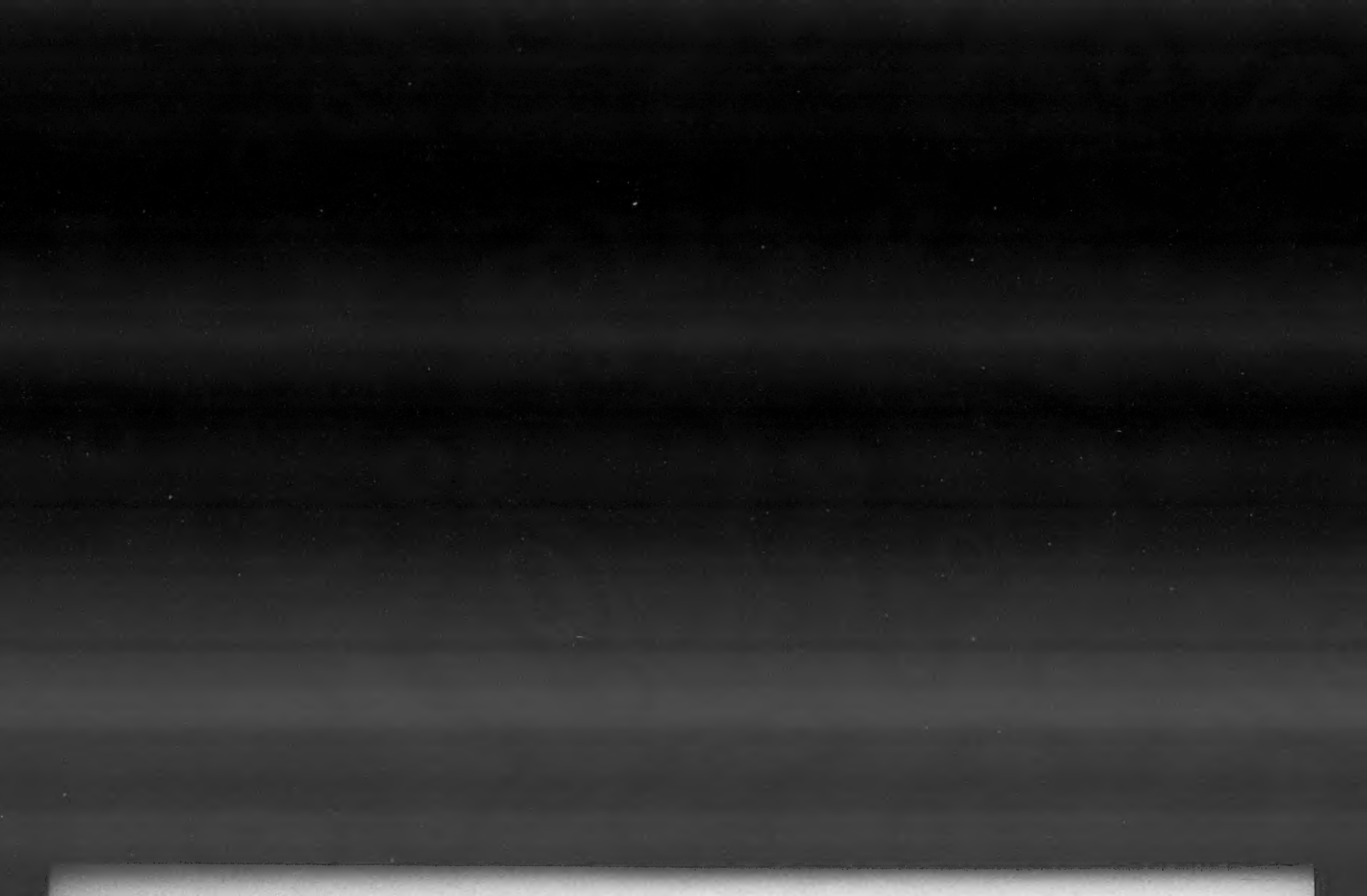
LEGENDS FOR FIGURES

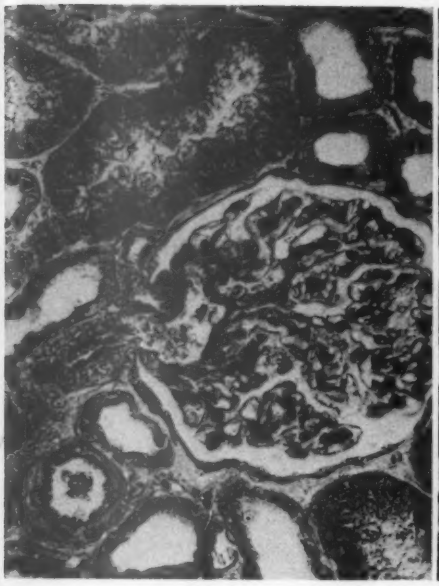
FIG. 1. Renal biopsy with grade II arteriolosclerosis. There is cloudy swelling of convoluted tubular epithelium with cytoplasmic granularity. Of note is the dilated larger arteriole at lower left. Hematoxylin and eosin stain. × 225.

FIG. 2. Dilated convoluted tubules with cloudy swelling in a kidney with grade II arteriolosclerosis and diffuse fibrinoid necrosis. Tubular changes appear to represent a more severe degeneration of the same general type shown in Figure 1. Hematoxylin and eosin stain. × 180.

FIG. 3. Collections of lymphocytes in interstitial tissue from a case with moderate renal arteriolosclerosis (grade II). Thickened arterioles and slightly dilated convoluted tubules are also shown. Hematoxylin and eosin stain. × 120.

FIG. 4. Dissolution of renal tubular basement membranes, associated with an accumulation of lymphocytes, from a case with grade II arteriolosclerosis. Sulfuric acid hematoxylin stain. × 850.





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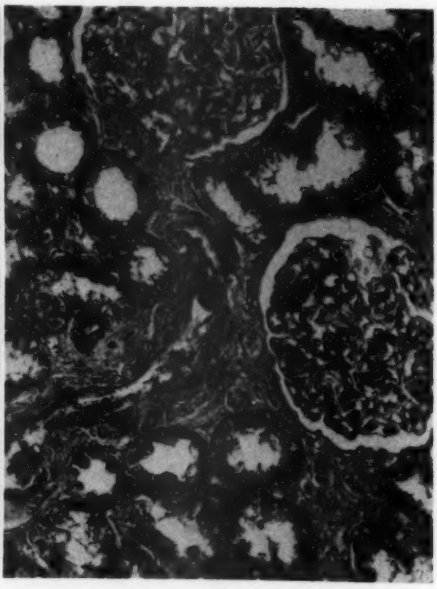

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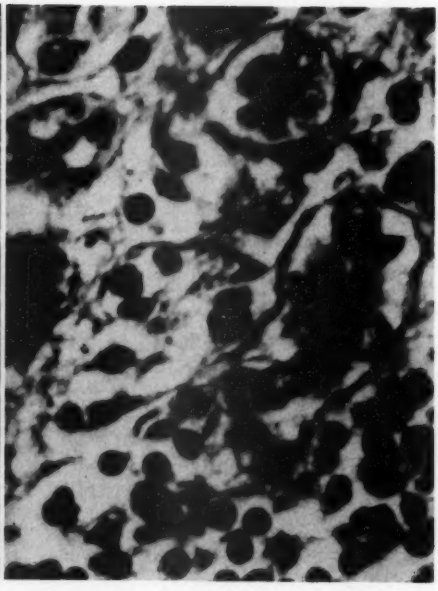

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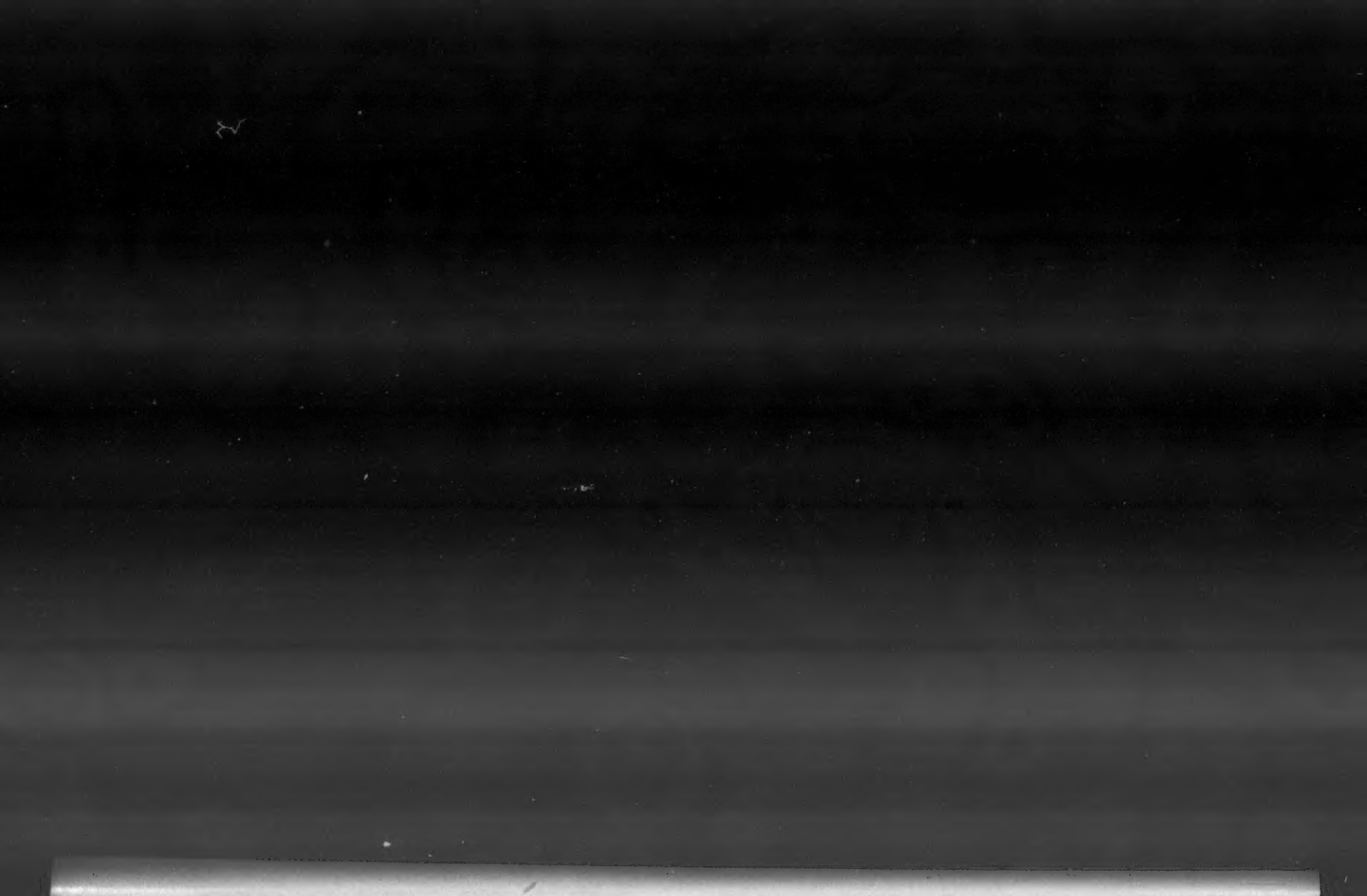
FIG. 5. Perivascular scars in a kidney biopsy with grade II arteriolosclerosis, showing moderate arteriolar thickening. Of note is the venous congestion. Hematoxylin and eosin stain. × 150.

FIG. 6. Slightly stiffened and thickened glomerular capillary walls, from a case with grade II arteriolosclerosis. Dilatation of a larger arteriole and vein are also shown. Verhoeff stain. × 180.

FIG. 7. Enlarged cells of the juxtaglomerular apparatus, with hydropic cytoplasm, in a kidney with grade I arteriolosclerosis. Verhoeff stain. × 300.

FIG. 8. Small groups of pale stained tubular cells, the so-called *Becher cells,* in a kidney with scars, cloudy swelling of convoluted tubules and grade II arteriolosclerosis. Phosphotungstic acid hematoxylin stain. × 150.



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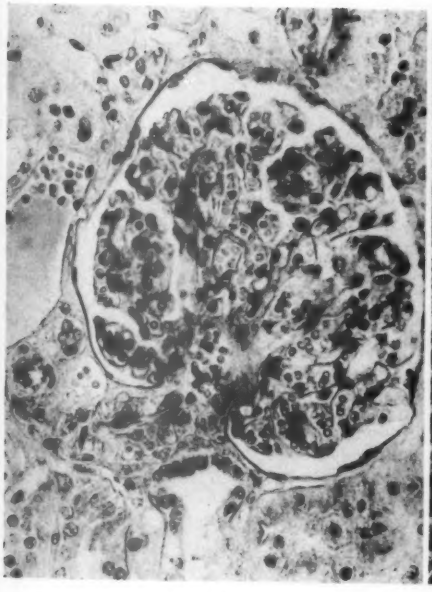

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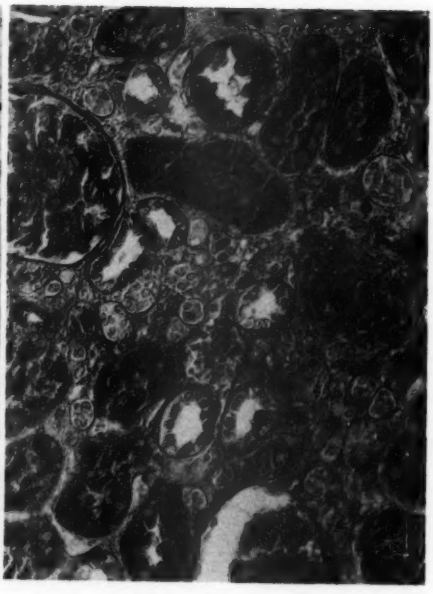

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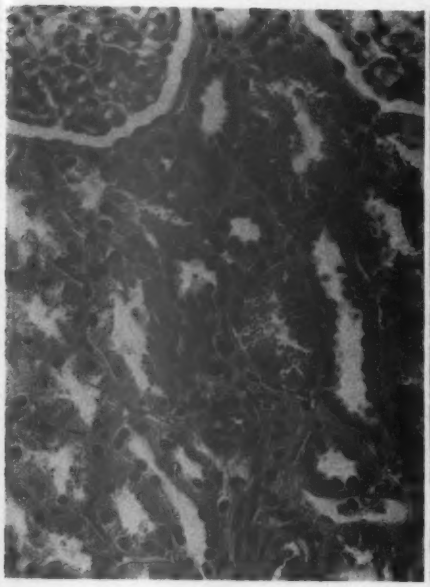
FIG. 9. Concentric overlapping of the arteriolar muscle cells indicative of spasm in otherwise normal arterioles from a kidney in hypertension. Hematoxylin and eosin stain. × 200.

FIG. 10. Hydropic change in the cytoplasm of muscle cells of an arteriole. There are prominent endothelium and enlargement of juxtaglomerular apparatus. Fine PAS-stained granules are present in some cells. Case with grade I-II arteriolosclerosis. Periodic acid-Schiff stain. × 200.

FIG. 11. Pooled PAS-positive material, partly shown as black subendothelial nodules in an arteriole. Grade I-II group. PAS stain. × 225.

FIG. 12. Stretched larger arteriole with a slightly thickened elastic membrane, and grade I arteriolosclerosis of a small arteriole. Tubular cloudy swelling is present. Aldehyde-fuchsin-PAS-orange-G stain. × 200.



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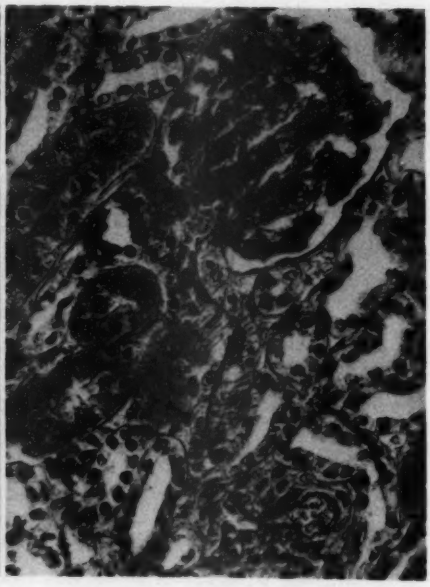

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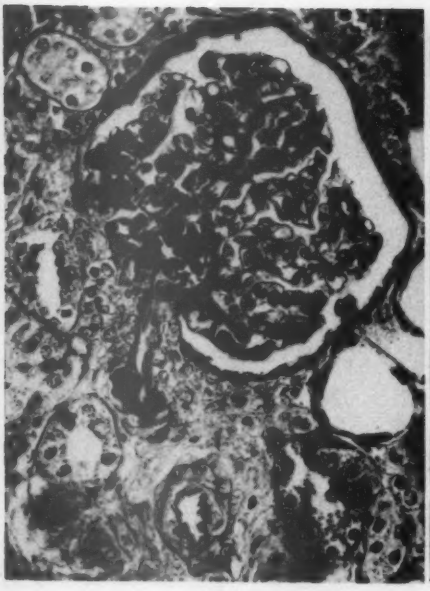

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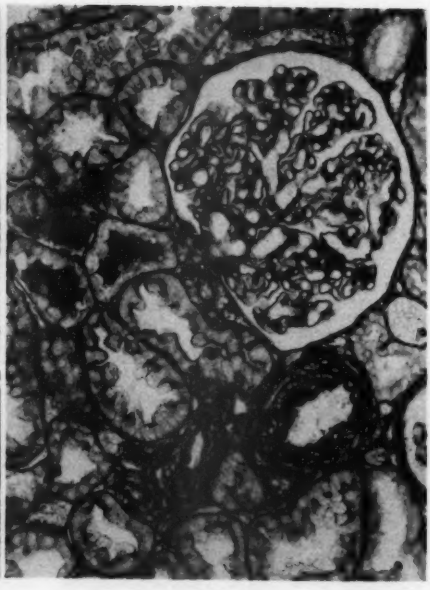

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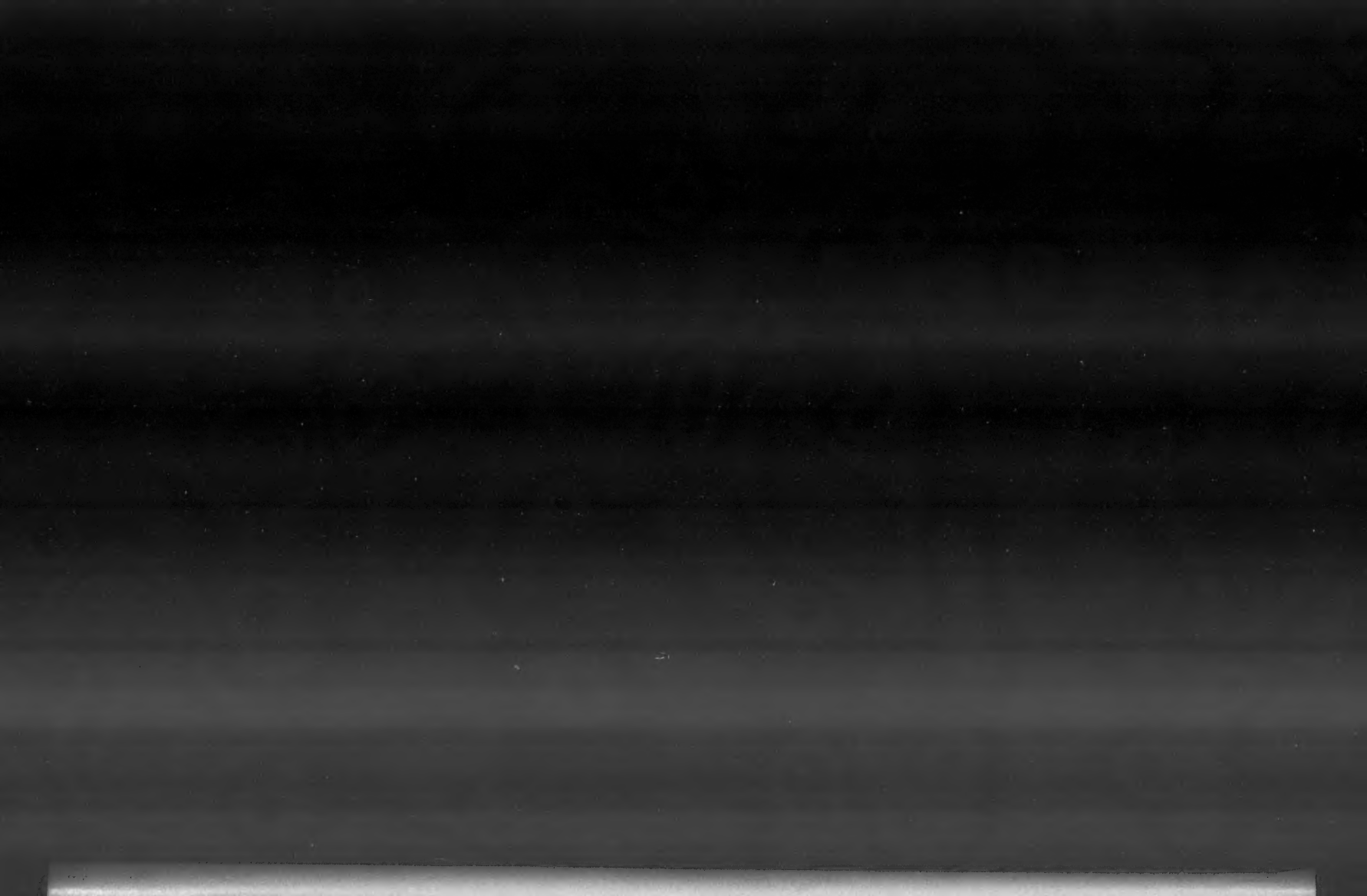
FIG. 13. Hypertrophy of an afferent arteriole, with prominent PAS-stained intercellular material shown in black. Case with grade II arteriolosclerosis. Glomerular ischemic changes are prominent. PAS stain. × 200.

FIG. 14. Another instance of arteriolar muscular hypertrophy, with prominent elastic tissue shown in black. Grade II arteriolar nephrosclerosis. Aldehyde-fuchsin-PAS-orange-G stain. × 150.

FIG. 15. More extreme hydropic changes and pooled PAS-positive material, shown in black, in renal arterioles with moderate arteriolosclerosis (grade II). PAS stain. × 150.

FIG. 16. Local collagen deposition, shown as the gray fibrillar portions of renal arteriolar walls which elsewhere have a moderate sclerosis (grade II) with pooled ground substance. PAS stain. × 200.



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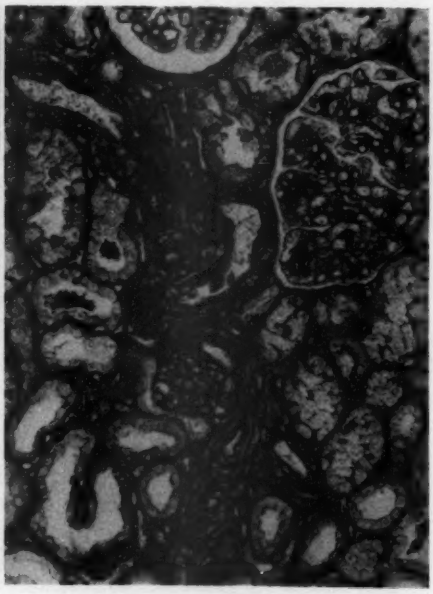

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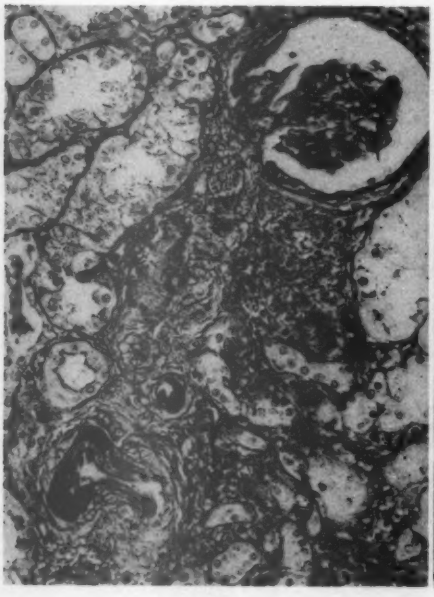

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FIG. 17. Severe thickening and degeneration of arterioles from a kidney with grade III arteriolosclerosis. Abundant PAS-positive (black) material is present. PAS stain. × 150.

FIG. 18. Splitting of the arteriolar elastic tissue and exaggerated amounts of pooled ground substance in severe renal arteriolosclerosis (grade III). PAS stain. × 200.

FIG. 19. Irregular degeneration of the larger arterioles, involving all major tissue components. Grade III arteriolosclerosis and fibrinoid necrosis of smaller arterioles. Aldehyde-fuchsin-PAS-orange-G stain. × 150.

FIG. 20. Variation in the degree of local thickening of larger renal arterioles, with localized collagen deposits, shown as gray fibrillar material. Grade III renal arteriolosclerosis. Masson stain. × 150.

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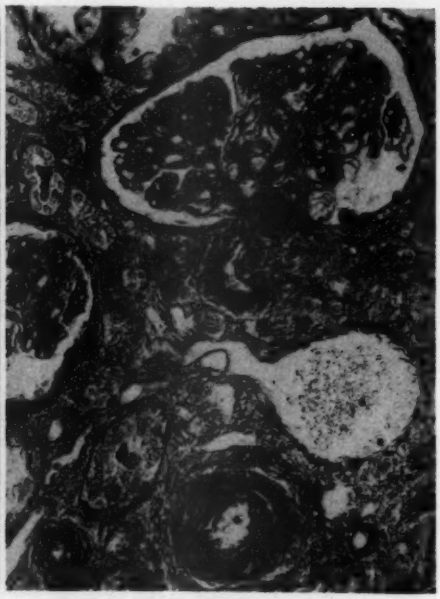

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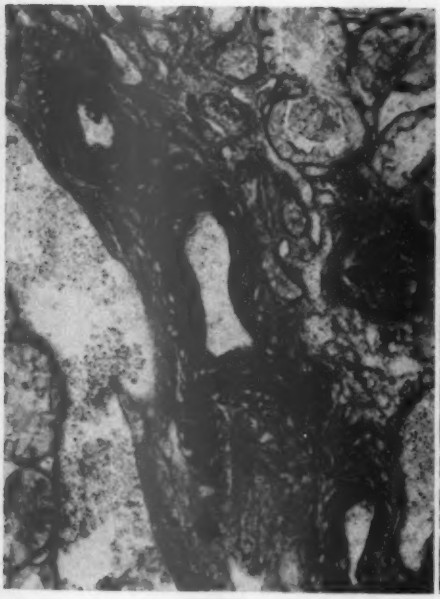

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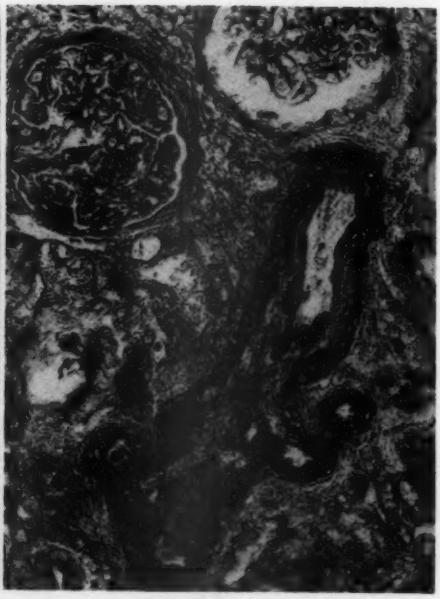

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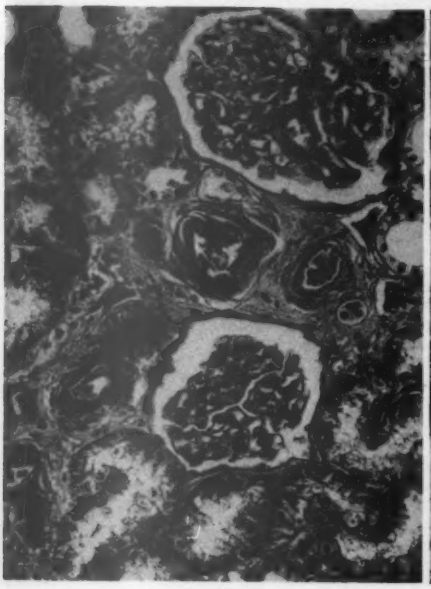
FIG. 21. Fibrinoid necrosis of small arterioles, with staining properties similar to the fibrin shown in a venule to left of center. The localization, irregular outlines, and staining characteristics favor its intramural deposition subsequent to imbibition of plasma. Grade II arteriolosclerosis, also shown in Figure 2. Hematoxylin and eosin stain. × 180.

FIG. 22. Foci of fibrinoid necrosis in grade III renal arteriolosclerosis, with a fibrin thrombus in the vessel at lower right. Phosphotungstic acid-hematoxylin stain. × 180.

FIG. 23. Acute and chronic pyelonephritis exhibiting a pus cast at lower right and interstitial leukocytic infiltrations, including plasma cells. Grade II arteriolosclerosis. Hematoxylin and eosin stain. × 120.

FIG. 24. Healed pyelonephritis with a flat-based scar of the cortical surface. Beneath this are colloid casts, scars, and moderate arteriolosclerosis. No plasma cells are identified. PAS stain. × 120.



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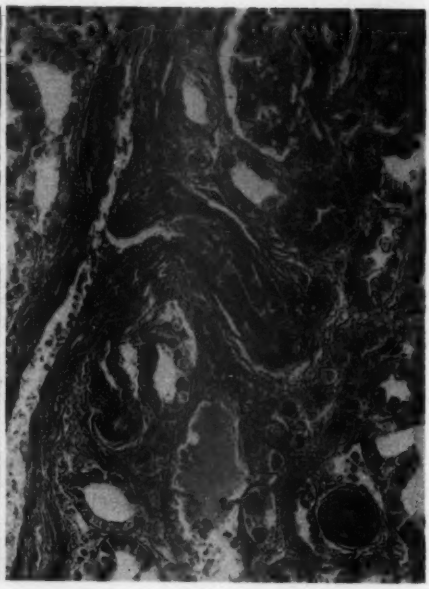

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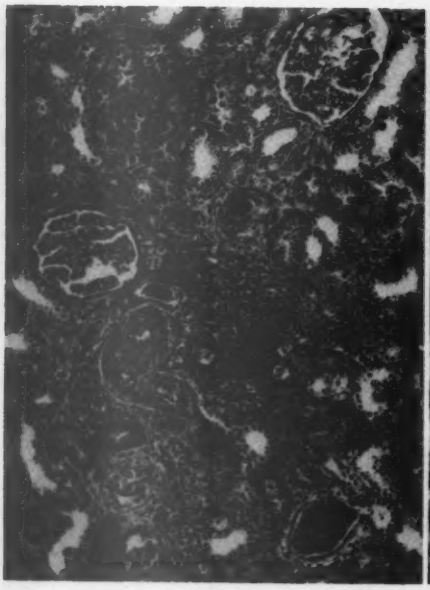

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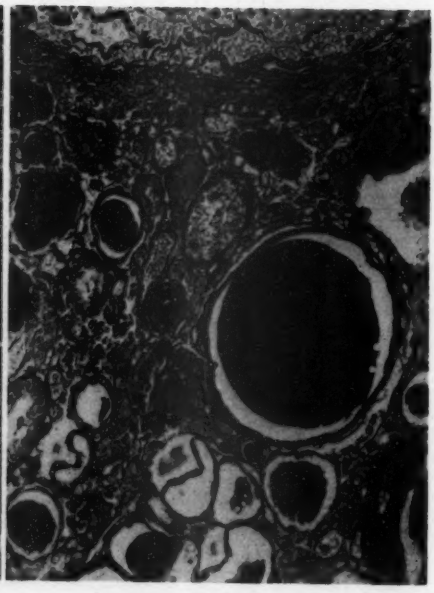

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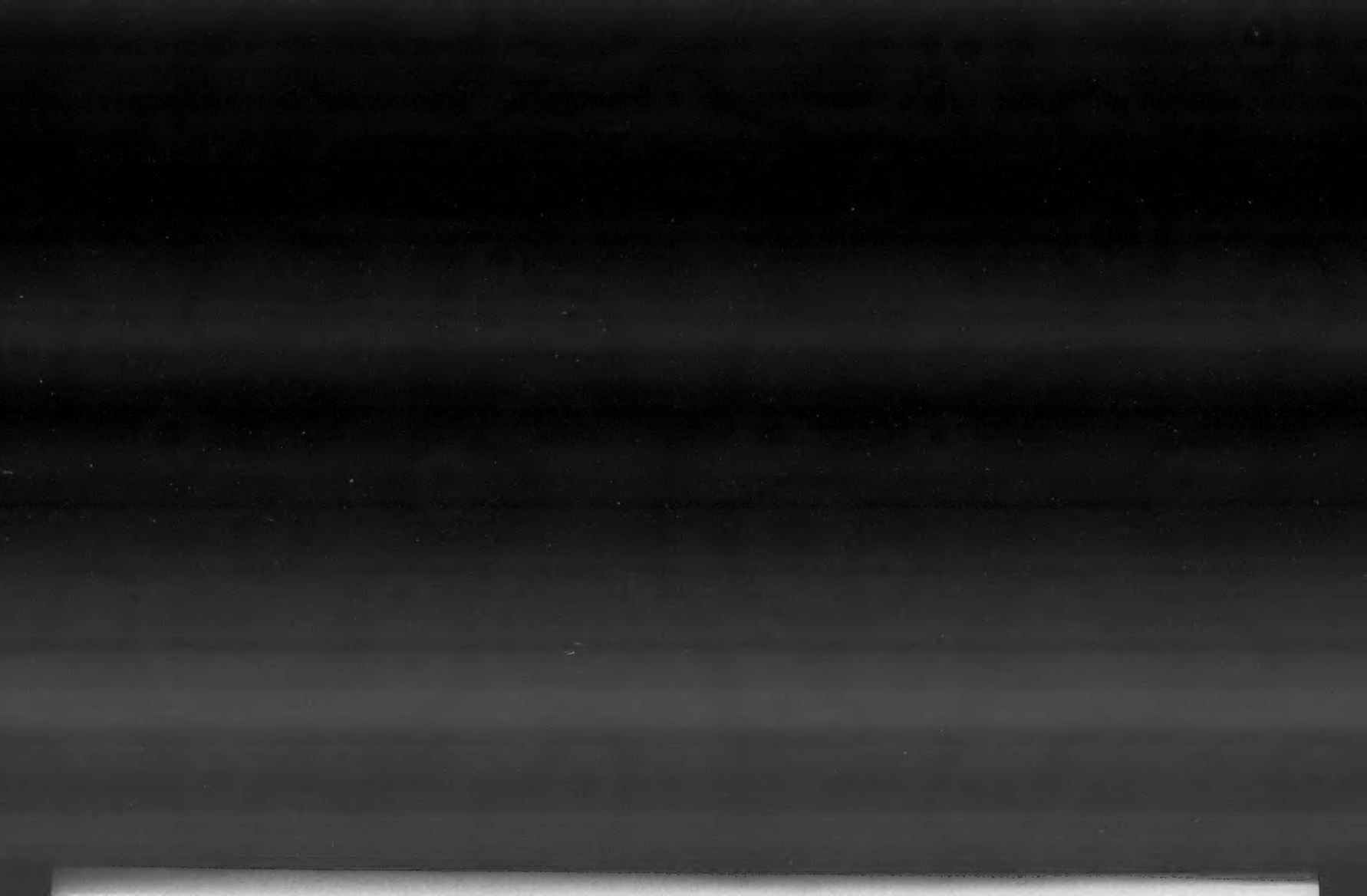
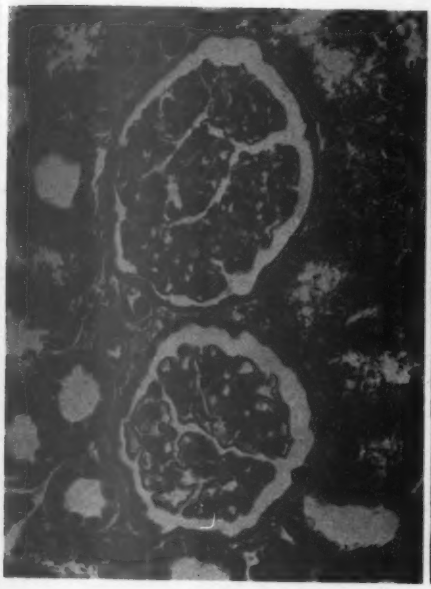
FIG. 25. Slight arteriolar sclerosis (grade I) in renal tissue procured at the time of removal of a pheochromocytoma accompanied by hypertension. Hematoxylin and eosin stain. × 180.

FIG. 26. Second kidney biopsy, from the same case shown in Figure 25, 6 years later. No arteriolar abnormality is evident. Hematoxylin and eosin stain. × 200.

FIG. 27. Membranous glomerulonephritis, with irregular and severe focal thickenings of basement membranes, as in the portion of glomerulus at lower right. This patient had grade I arteriolosclerosis. Hematoxylin and eosin stain. × 225.

FIG. 28. Interstitial collections of foam cells in a specimen with grade I arteriolosclerosis in one kidney and grade III sclerosis and severe pyelonephritis in the other. Case mentioned in the text as a possible example of the Goldblatt phenomenon. There is pyelonephritis in the area illustrated. Hematoxylin and eosin stain. × 150.





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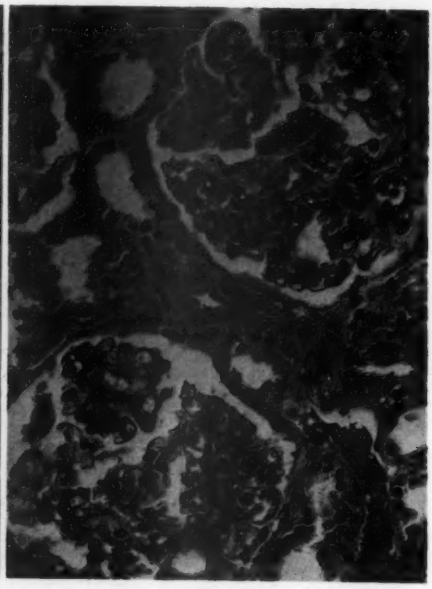

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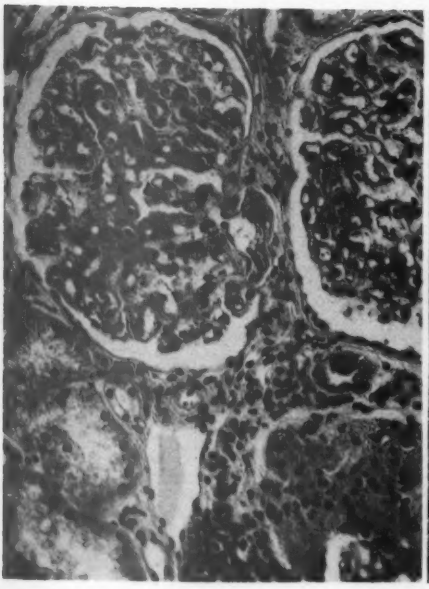

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FUNCTIONAL AND MORPHOLOGIC CHANGES IN HEART LUNG PREPARATIONS FOLLOWING ADMINISTRATION OF ADRENAL HORMONES *

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An increased incidence and severity of medial arteriosclerosis in rabbits was reported by Josué[1] following repeated injections of epinephrine. These observations have been confirmed by other investigators.[2-6] More recently Waters and de Suto-Nagy[7] reported that repeated intravenous infusions of large amounts of epinephrine (100 μg. per kg.) in the dog produced widespread, severe vascular lesions, with a preponderant location in the cardiac vessels. Vishnevskaya[8] also reported myocardial lesions in rats after a single intramuscular injection of 0.5 mg. of adrenalin. He attributed these lesions to a reflex mechanism, since he stated that they were not present in spinal preparations. King[9] also reported structural changes in the heart, liver, and kidneys of dogs given a single intravenous injection of adrenalin or noradrenalin (5 μg. to 30 μg. per kg. of body weight). The gross cardiac lesions consisted of extensive subendocardial and epicardial hemorrhages, areas of myocardial infarction, and extreme valvular edema. In view of the widespread metabolic and physiologic alterations which follow epinephrine or norepinephrine administration, it was not possible to link the observed cardiac lesions directly with the sympathomimetic amines themselves. The present series of experiments was devised to observe the influence of the sympathomimetic amines on the heart-lung preparation, with the heart isolated from all secondary hormonal or nervous influences.

METHODS

Experimental System

Thirty heart-lung preparations (HLP) were completed, using mongrel dogs of similar age and weight. The conventional heart-lung preparation of Starling[10] was modified only slightly for the purpose of

* This work was supported in part by grants from the Minnesota Heart Association, the U.S. Public Health Service (H-2540), and the Graduate School of the University of Minnesota.

Received for publication, November 21, 1957.

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this study. The venous blood reservoir, constructed of thin plastic sheeting, was filled with approximately one liter of heparinized blood. The temperature of the blood was maintained constantly to within ± 1° C. by a thermostatically controlled heating plate which was placed under the water bath surrounding the reservoir. Ventilation of the lungs was effected by a constant inspiratory pressure electronic respirator which permitted control of variation of the volume and rate of ventilation. A mixture of 5 per cent CO_2 in O_2 was utilized as the ventilating gas because its use ordinarily permitted maintenance of arterial pH at 7.40 ± 0.03. The filling level of the venous reservoir was kept constant throughout the experiment (10 cm. of water), and the Starling resistance to arterial outflow was maintained at approximately 100 mm. of Hg.

Experimental Procedure

Approximately 1½ liters of donor blood were withdrawn through a carotid artery cannula from a dog under local cervical anesthesia (100 mg. procaine hydrochloride) after the animal had been paralyzed with succinyl dicholine chloride (Anectine,® 1.0 mg. per kg. of body weight). This method of anesthesia was employed in an attempt to reduce to a minimum the circulatory depressant effects of barbiturates. During this time the donor dog was ventilated mechanically.

The heart-lung preparation was established in a dog anesthetized with pentobarbital (Nembutal,® 25 mg. per kg. of body weight), paralyzed with Anectine,® and mechanically ventilated with air until the preparation was complete. A mixture of 5 per cent CO_2 in O_2 was then used throughout the experiment. Inflow and outflow pressures were recorded continuously from 20-gauge needles placed in the superior vena cava cannula and brachiocephalic outflow cannula respectively, in turn connected to strain gauges and a direct pen recording Sanborn Poly-Viso instrument. Cardiac output was measured with a flow meter.

In 8 control experiments nothing was done to the preparation. In 7 experiments, 10 minutes after completion of the preparation, 20 μg. of epinephrine hydrochloride (Lederle injection solution) or norepinephrine bitartrate (Levophed®) was infused into the superior vena cava at a constant rate of 5 μg. per minute. Seven other preparations received 10 mg. of hydrocortisone or 2.5 mg. of delta hydrocortisone in a single injection, and 8 received the same dose of corticoid, followed 10 minutes later by a constant infusion of 20 μg. of epinephrine or norepinephrine.

Sixty to 70 minutes following completion of the preparation, the superior vena cava was occluded, the heart was sectioned at the base

of the large vessels and removed from the chest. The experiments were completed before any significant signs of dilatation of the heart appeared in the preparation.

Analytic Procedure

Mean arterial blood pressure was measured and cardiac work calculated, using the relation: $W = P \times F$; P = Mean systemic pressure in meters of H_2O; F = Flow in cc. per minute; W = Work in gm.—meters per minute.

After macroscopic examination, the hearts were fixed in 10 per cent neutral formalin. Several blocks from each heart were sectioned for microscopic study. These included specimens from the myocardium and septum, the valves, and the root of the aorta. The sections were stained with hematoxylin and eosin; additional sections were stained by the periodic acid-Schiff (PAS) method.

Results

Physiologic Observations

The changes observed in the physiologic variables are illustrated in Text-figures 1, 2, and 3.* There were progressive, spontaneous alterations in the control heart-lung preparations over a one hour period. The most striking change was a fall in heart rate, associated with parallel decrease in cardiac output and minute work. After a constant infusion of epinephrine or norepinephrine (5 μg. per min.) an increase in heart rate, output and work was observed. This rise was observed a few seconds following initiation of the infusion, and reached a plateau within one minute. The effect of the sympathomimetic amine injected in this manner was prolonged, and lasted 10 to 15 minutes after the infusion was discontinued.

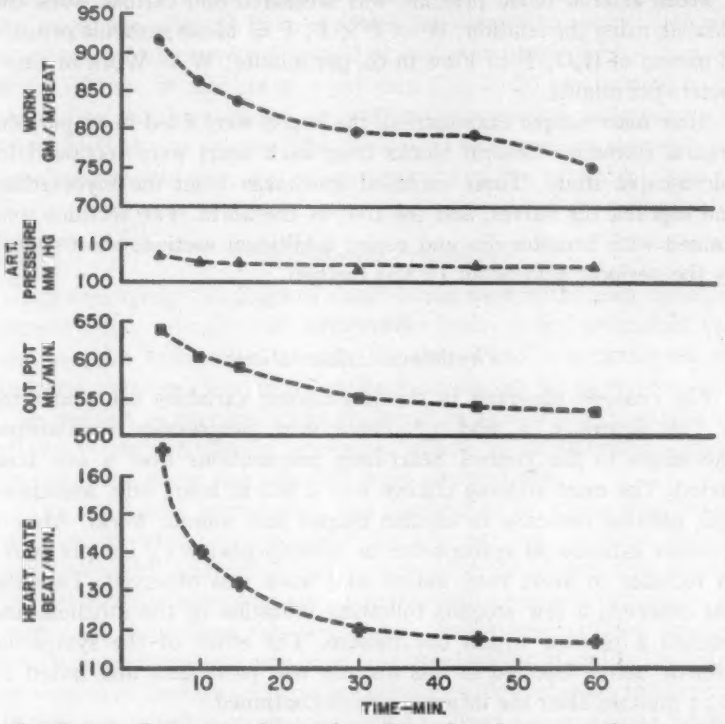
The addition of 10 mg. of hydrocortisone succinate to the preparation had no chronotropic or inotropic effect on the heart. When the administration of hydrocortisone succinate (Text-fig. 3) preceded the infusion of sympathomimetic amines, the stimulating action of the latter was more marked in some preparations.

Morphologic Observations

Upon gross examination, the hearts showed extensive subepicardial and subendocardial hemorrhages (Fig. 1). The tricuspid, mitral, and aortic valves were edematous and appeared distorted. The changes

* The Text-figures were prepared by the Medical Audio Visual Department, Walter Reed Army Institute of Research, Washington, D.C.

were more marked along the free margins of the valves, although hemorrhages were noted occasionally in their bases. The degree of valvular edema was occasionally so marked that the entire leaflet appeared as a large, gelatinous mass of tissue. These lesions were more pronounced in the preparations in which cortisone was admin-



Text-figure 1. Spontaneous changes observed in a heart-lung preparation over a one hour period.

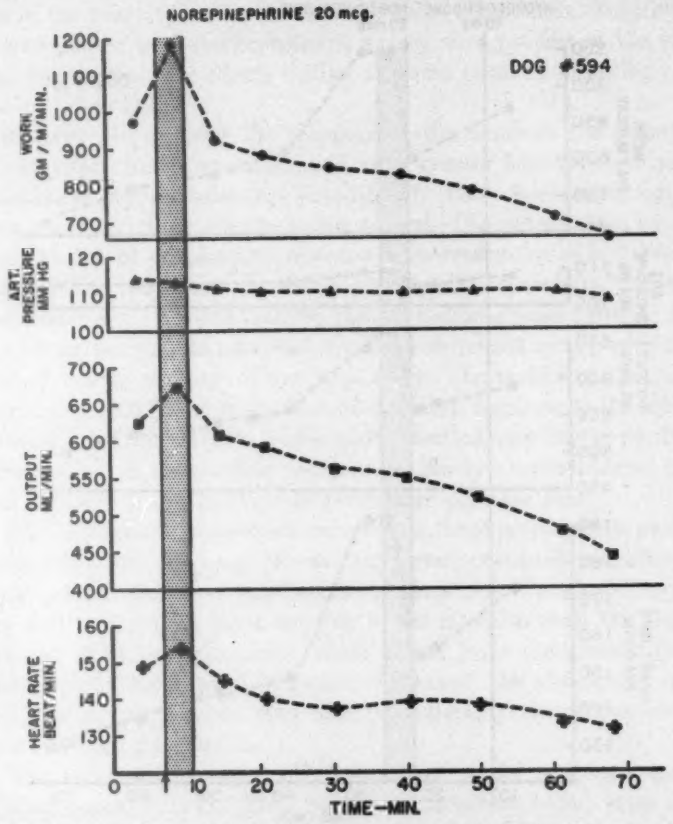
istered with either epinephrine or norepinephrine. No changes were observed in the pulmonary valves. In one control animal subepicardial hemorrhage was present, but the valves appeared normal.

Microscopic examination of the hearts disclosed extensive subendocardial and subepicardial hemorrhages (Fig. 2) as well as hemorrhage and edema in the myocardium (Fig. 3). In some sections foci of frank myocardial infarction were observed and were associated occasionally with hyaline thrombi in the intramural coronary arteries. In the walls of other coronary arteries, scattered segmental deposits of hyaline fibrinoid material were noted. These lesions were accompanied by perivascular edema and hemorrhage.

Microscopically, the valvular changes consisted of marked edema

and disruption of valve substance accompanied by varying amounts of hemorrhage and deposits of fibrinoid material. The edematous tissue varied in tinctorial properties from a distinct acidophilia to a slight basophilia (Figs. 4 to 6).

These alterations were observed in all of the experimental hearts

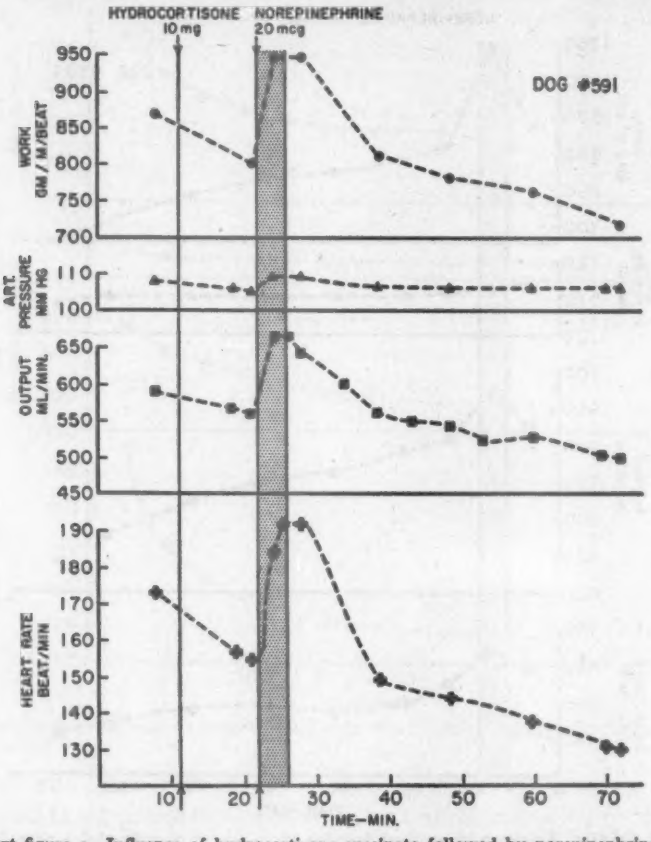


Text-figure 2. Influence of constant infusion of 20 μg. of norepinephrine on a heart-lung preparation.

in which cortisone, epinephrine or norepinephrine were used, but were more extensive and severe in the preparations in which these substances were used in combination. In the control preparations, only one heart exhibited any gross or microscopic alteration. This consisted of a moderate degree of subepicardial and myocardial hemorrhage in the region of the catheter.

In addition to the cardiac changes, another lesion was observed in those preparations in which both cortisone and epinephrine or nor-

epinephrine were introduced. This consisted of disruption and fragmentation of the elastic fibers in the root of the aorta, and was accompanied by cystic lesions containing a fibrillar material which was slightly basophilic in staining. Scattered deposits of fibrinoid material were also observed in these lesions. The alterations were more promi-



Text-figure 3. Influence of hydrocortisone succinate followed by norepinephrine on a heart-lung preparation.

nent near the intimal surface of the aorta, but in some animals they extended approximately half way through the media (Fig. 7). The lesions bore a resemblance to human medial cystic aortic necrosis.

Discussion

The cardiac lesions present in the preparations in which the sympathomimetic amines were administered are similar in nature and distribution to those which have been described in intact animals

receiving similar or larger doses of epinephrine or norepinephrine intravenously.[9] However, with a similar dosage, the lesions are much more extensive and severe in the isolated heart. A possible mechanism of action in the production of such lesions is the damaging effect of the prolonged increase in myocardial tension and of the rapid pulsation of the heart under the influence of these compounds. Inactivation of epinephrine or norepinephrine is a very slow process in the heart-lung preparation, the effects lasting 15 to 20 minutes following cessation of administration.

In some preparations the response of the heart to the same dose of sympathomimetic amine seemed to be greater following the administration of hydrocortisone (Text-fig. 3). This observation has also been made by others on the intact animal. The potentiation of corticoids on the blood pressure response to norepinephrine in adrenalectomized dogs has been reported by Ramey and co-workers.[11] Kurland and Freedberg[12] showed that the pressor response to an infusion of 2.1 to 13.8 μg. per min. in man was strikingly increased by the administration of 150 to 200 mg. of cortisone daily. The results of the present experiments suggest that the increased pressor response to the injection of norepinephrine after cortisone administration may be due partly to a potentiation of the cardiac response to the sympathomimetic amine, and not only to an increase in peripheral vascular tone.

The dosages of corticoid administered in these preparations produced a concentration of 5 μg. per cc. with hydrocortisone succinate, and 1 μg. per cc. with delta hydrocortisone succinate. Such concentrations are well in excess of those existing in the intact animal, but they are not too different from those which result from therapeutic dosages administered for clinical purposes. However, the absence of normal inactivating mechanisms may alter considerably the response of the heart to such compounds.

The structural changes which occurred in the heart preparations did not appear to impair their functional properties to any great extent. No signs of acute, accelerated failure which could have been differentiated from their spontaneous, slow deterioration were observed in any of them.

Many of the hemorrhagic lesions could have been the result of the mechanical forces to which the vessels were subjected. As there exists in each vessel a critical closing pressure, there also exists a "critical distending pressure" beyond which the media of the vessel will rupture. Byrom and Dodson[13] suggested a direct relationship between increased intra-arterial pressure and necrosis of renal arterioles. However, the rise in pressure observed in the heart-lung preparations after epineph-

rine or norepinephrine administration, never exceeded 40 mm. of Hg, and such an increment in pressure alone could not account for the extensive lesions observed. These experiments indicate that, in the absence of normal inactivating mechanisms, small dosages of sympathomimetic amines will produce extensive vascular lesions in the isolated heart.

The influence of the sympathomimetic amines on lipid metabolism and transport in the intact animal has been established.[14,15] Dury[14] reported that the ratio of free cholesterol to total cholesterol increased in rabbits given daily multiple injections of epinephrine. It is possible that the lipids so mobilized would tend to deposit in the areas of mechanical vascular damage also induced by the sympathomimetic amines. The mechanism of production of the valvular edema is less clear. The dilatation and engorgement of the lymphatics, which was most noticeable in the liver of intact dogs receiving intravenous injections of sympathomimetic amines,[9] suggest that alterations in lymphatic flow may have some bearing on the production of this edema.

Summary

Thirty heart-lung preparations were completed using mongrel dogs of similar age and weight. Total weight of each of the preparations was 2 kg. Cardiac output, outflow pressure, and the electrocardiogram were continuously recorded. Seventy minutes after completion of the preparation, inflow was clamped and the heart was rapidly excised. Eight preparations were used as controls, and 7 received 4 μg. of epinephrine or norepinephrine per minute for 5 minutes. Seven other preparations received 10 mg. of hydrocortisone in a single injection, and in 8, hydrocortisone administration was followed 10 minutes later by the injection of epinephrine or norepinephrine. In the control series, cardiac work, output, and rate fell slightly and gradually in the course of the 70-minute experiments. No macroscopic or microscopic alterations were observed after removal of these hearts, except in the immediate vicinity of the intracardiac caval cannulae.

The infusion of sympathomimetic amines caused a marked rise in cardiac work and rate which dropped below their control values after cessation of the infusion. Hydrocortisone alone did not cause any significant alteration in functional activity. However, all the hearts receiving one of the adrenal hormones presented extensive lesions of the myocardium, valves, and coronary vessels. The lesions were more severe in the hearts in which both adrenal hormones were used.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All sections were stained with hematoxylin and eosin.

FIG. 1. Gross photograph of a heart showing the opened left ventricle. There is extensive subendocardial hemorrhage and edema of the aortic valve cusps.

FIG. 2. Extensive hemorrhage beneath the endocardium of the left ventricle. From a preparation to which 20 μg. of norepinephrine was administered. × 70.

FIG. 3. Section of the myocardium from another heart-lung preparation which shows a large area of muscle necrosis and hemorrhage. Twenty μg. of epinephrine was administered. × 70.

The gross photograph was made by the Medical Audio Visual Department, Walter Reed Army Institute of Research. The photomicrographs were made by Mr. Henry Morris.

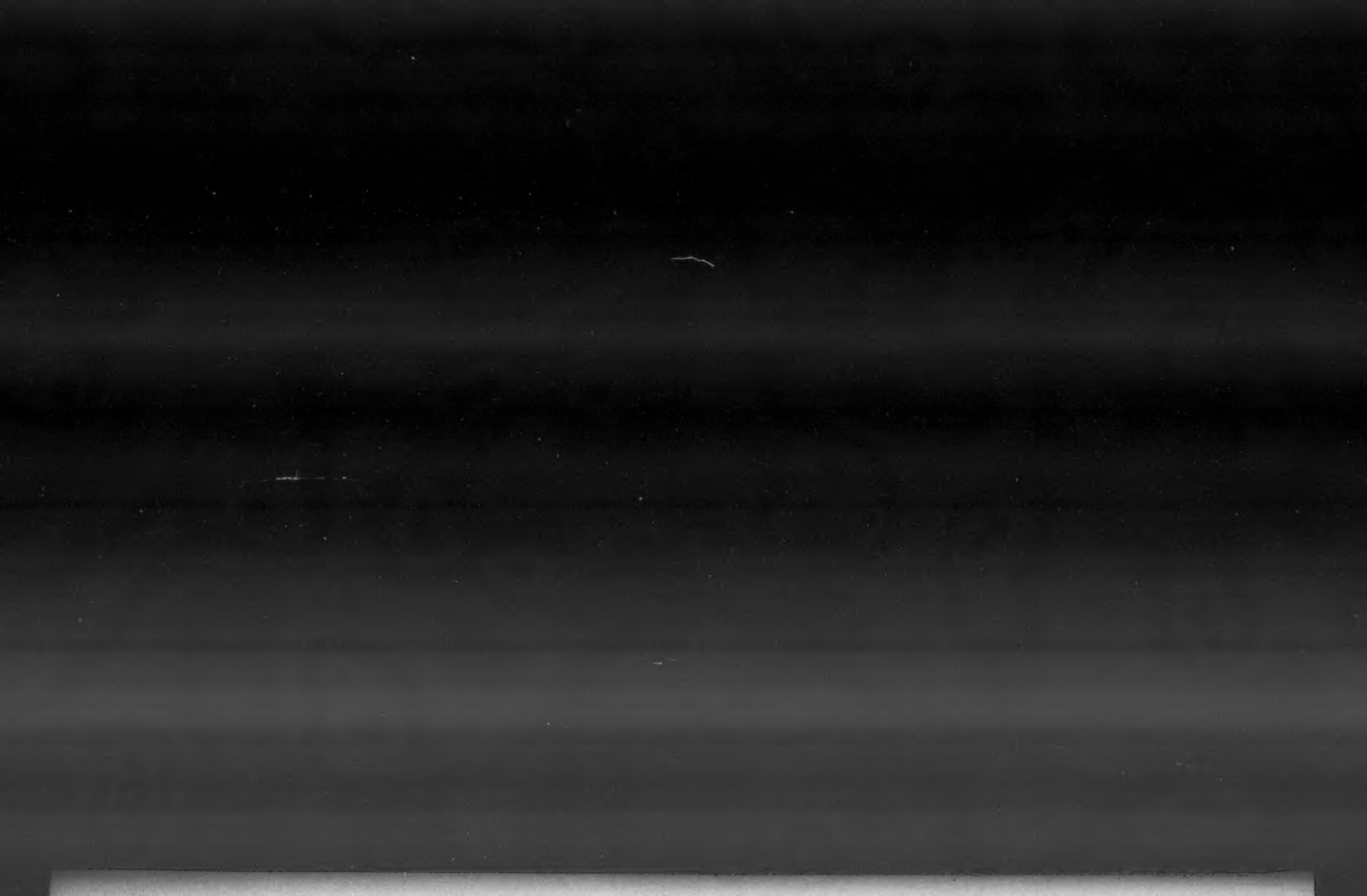

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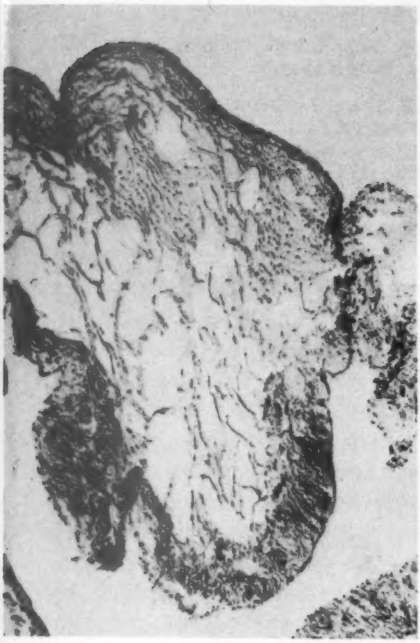
FIG. 4. Section of the tricuspid valve. There is marked edema and disruption of the substance of the valve. Ten mg. of hydrocortisone and 20 μg. of norephinephrine administered. × 70.

FIG. 5. Section of the aortic valve from a preparation to which 10 mg. of hydrocortisone was administered. The valve shows considerable edema and hemorrhage. × 70.

FIG. 6. Section of the mitral valve from an isolated heart-lung preparation in which 20 μg. of epinephrine was used. Severe, extensive hemorrhage is present throughout the substance of the valve. × 70.

FIG. 7. Section from the root of the aorta which shows disruption and fragmentation of the elastic fibers, and the presence of cystic lesions which contain fibrillar, slightly basophilic material. Heart-lung preparation; 10 mg. of hydrocortisone and 20 μg. of norepinephrine administered. × 70.



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THE NATURAL HISTORY OF ATHEROSCLEROSIS
COMPARISON OF THE EARLY AORTIC LESIONS IN NEW ORLEANS, GUATEMALA, AND COSTA RICA*

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From the Louisiana State University School of Medicine, Charity Hospital of Louisiana at New Orleans, La., and the Institute of Nutrition of Central America and Panama (INCAP), Guatemala City, Guatemala.

It is widely believed that there is a marked difference in the incidence of clinical disease due to atherosclerosis in the United States and in certain other countries, particularly with respect to myocardial infarction.[1-9] Satisfactory data to substantiate this impression are difficult to obtain, but the belief is so strong that many investigators have sought other differences between the various populations, which may be related to, and possibly responsible for, the difference in incidence of disease attributable to atherosclerosis. Those factors which have been most extensively studied have been diet, serum cholesterol levels, and concentrations of other serum lipids, principally lipoproteins.[10-12] However, practically no information has been accumulated which would permit a systematic comparison of the degree of arterial system involvement by atherosclerosis in these populations. The difficulty of collecting suitable anatomic material and the lack of acceptable methods for quantitating the lesions are principally responsible for the paucity of such data.

In previous reports, there have been described practical methods for collection, fixation, and storage of arterial specimens for future reference. A method of quantitatively estimating the extent of atherosclerotic lesions in them has also been established.[13-17] These techniques, applied to aortic specimens collected in New Orleans, have demonstrated significant differences between the Negro and white races in certain types of lesions at certain ages. Furthermore, a base line has been established for lesions measured in this manner in the New Orleans population, with which other groups of specimens measured in a similar fashion may be compared.

While this work was being carried on in New Orleans, a study was begun independently in Guatemala and Costa Rica by the Institute of Nutrition of Central America and Panama, using similar but not identical techniques. The methods and results from the Institute have

* These studies were aided by grants from the Louisiana Heart Association and the National Heart Institute of the United States Public Health Service (H-655, H-1785, and H-2653).

Received for publication, December 18, 1957.

been published previously.[9,15] Part of this material is used in the present report.

Collaboration between the two laboratory groups and the sharing of specimens collected in the different areas now permit a comparison of data derived from investigation of the Guatemalan and Costa Rican lesions with that gathered in New Orleans. The emphasis in these studies has been on the early stages of atherosclerosis, before the development of hemorrhage, ulceration, or thrombosis, and before the production of clinical manifestations.

Materials and Methods

All aortas were fixed, stained grossly with Sudan IV, and preserved in plastic bags. The extent of the atherosclerotic lesions was graded in terms of per cent of intimal surface covered by each of 3 types of lesions: fatty streaks, fibrous plaques, and "complicated lesions." The techniques utilized and the definitions and illustrations of the types of lesions have been described previously.[16,17] All estimates of the lesions referred to in this report were made by two individuals (JPS and HCM). Consid-

Table I
Tabulation of 800 Cases Investigated

Age in years	New Orleans—White			New Orleans—Negro			Guatemala			Costa Rica			Total
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	
1 – 5	18	11	29	54	43	97	13	12	25	20	18	38	189
6 – 10	12	3	15	15	11	26	11	4	15	2	3	5	61
11 – 15	8	5	13	14	9	23	10	2	12	5	4	9	57
16 – 20	11	6	17	20	8	28	11	4	15	4	3	7	67
21 – 25	19	5	24	14	12	26	21	16	37	3	4	7	94
26 – 30	12	9	21	13	22	35	22	7	29	11	5	16	101
31 – 35	8	8	16	14	15	29	25	18	43	9	7	16	104
36 – 40	14	13	27	18	17	35	35	15	50	5	10	15	127
Total	102	60	162	162	137	299	148	78	226	59	54	113	800

erable experience and practice is usually necessary to obtain consistent results in the grading of lesions by different individuals.

The specimens analyzed consisted of 461 aortas from the New Orleans area (299 of these were from Negroes, and 162 were from whites); 226 aortas from Guatemala; and 113 aortas from Costa Rica. The cases are divided by age and sex in Table I. All of the specimens were from patients between 1 and 40 years of age and were accompanied by brief clinical and necropsy data. In New Orleans, the specimens were obtained from consecutive necropsies performed at the Charity Hospital of Louisiana and at the Office of the Coroner, Parish of Orleans. In Guatemala, the specimens were obtained from necropsies at the General Hospital; the patients were predominantly of Mayan Indian extraction. In Costa Rica, the specimens were obtained from necropsies performed at the San Juan de Dios Hospital, San José; these patients were of almost pure Spanish white descent. All 3 hospitals are large, general, charity hospitals serving lower income groups, and would be expected to furnish necropsy populations bearing as nearly the same relationship to the living population as it is possible to obtain.

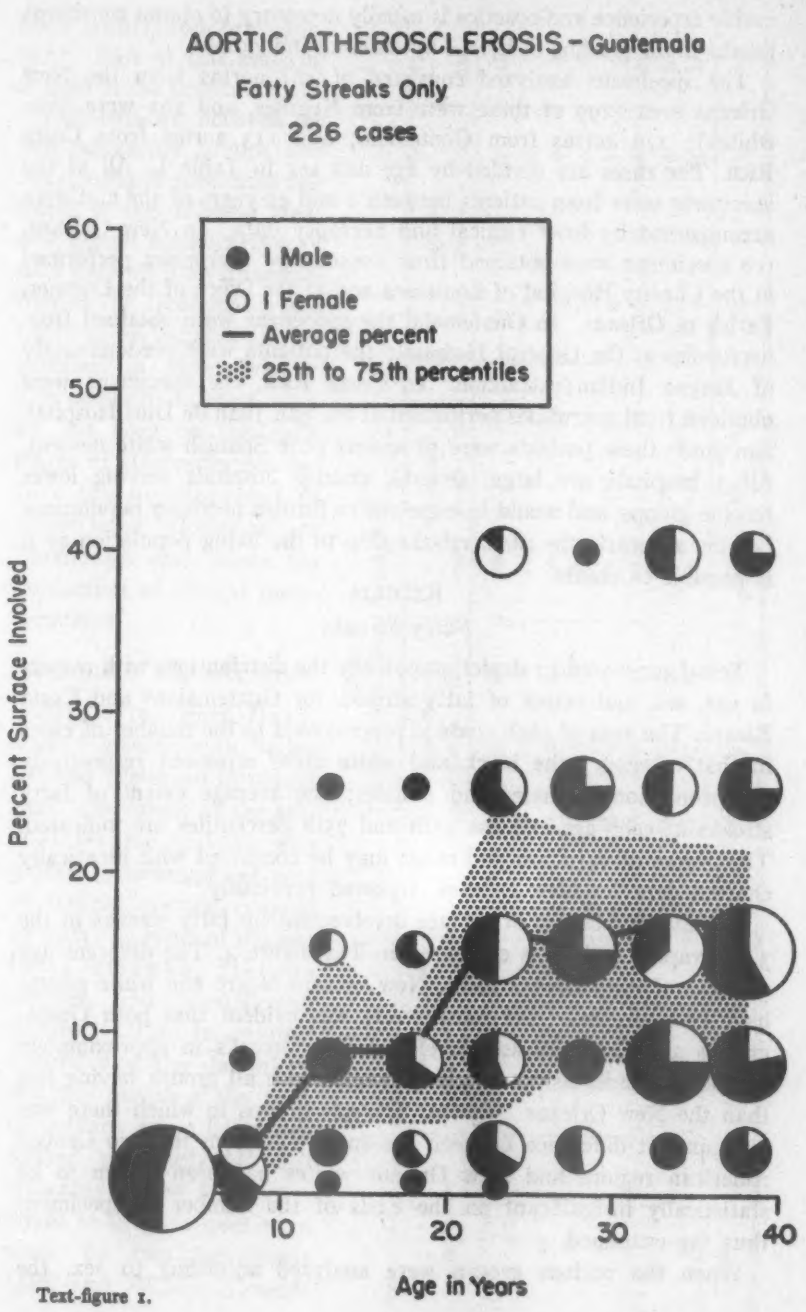
Results

Fatty Streaks

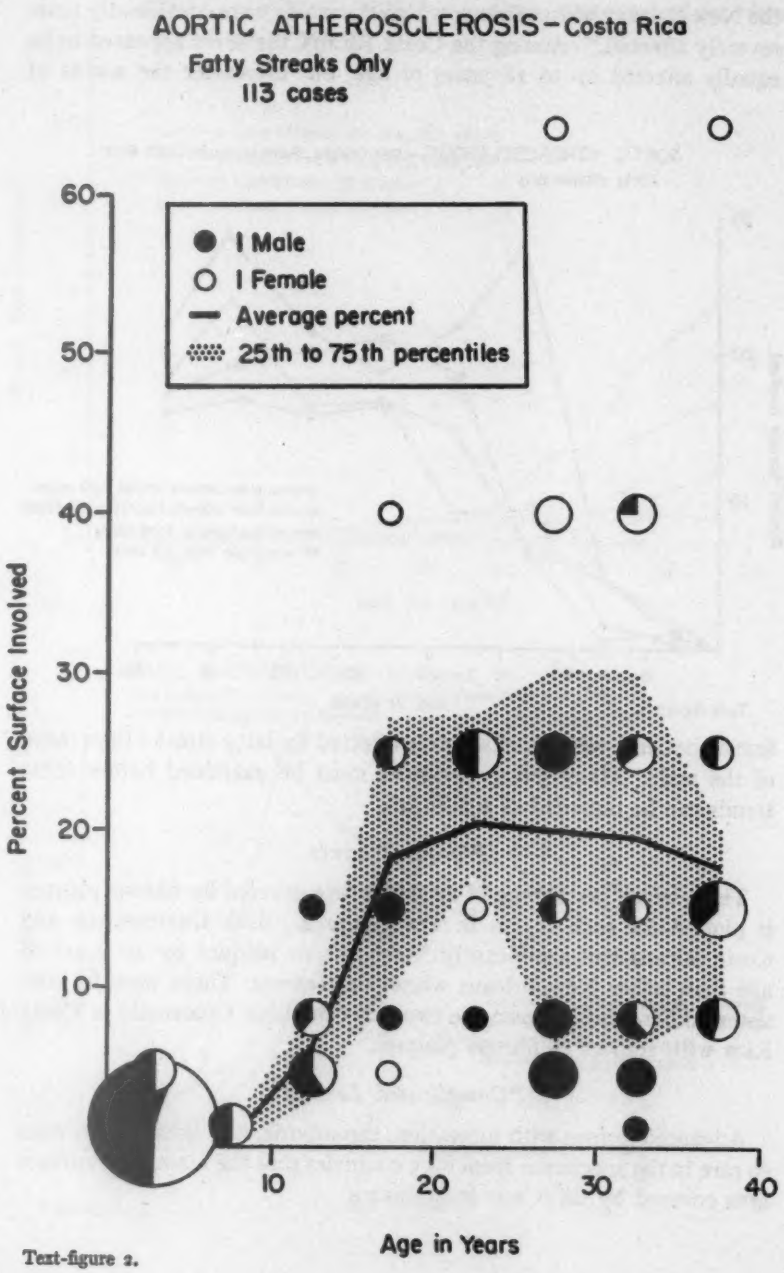
Text-figures 1 and 2 depict graphically the distributions with respect to age, sex, and extent of fatty streaks for Guatemalans and Costa Ricans. The area of each circle is proportional to the number of cases in that category; the black and white areas represent respectively the proportion of males and females; the average extent of fatty streaks at each age and the 25th and 75th percentiles are indicated. The degree of variation and range may be compared with identically charted data for New Orleans, reported previously.[16]

The average degree of surface involvement by fatty streaks in the 3 geographic regions is compared in Text-figure 3. The different age patterns of fatty streaks in the New Orleans Negro and white groups have been discussed previously.[16] It was evident that both Guatemalans and Costa Ricans developed fatty streaks in approximately the same manner as New Orleans whites with all groups having less than the New Orleans Negroes. The age groups in which there was an apparent difference between the specimens from the two Central American regions and New Orleans whites has been shown to be statistically insignificant on the basis of the number of specimens thus far examined.

When the various groups were analyzed according to sex, the

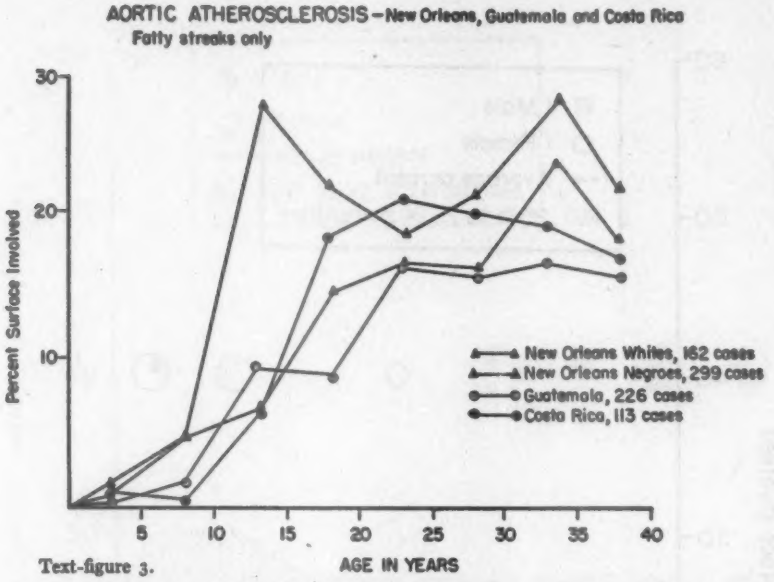


Text-figure 1.



Text-figure 2.

Guatemalans showed no significant difference between the sexes, unlike the New Orleans whites among whom the males were consistently more severely affected.[16] Among the Costa Ricans, the sexes appeared to be equally affected up to 18 years of age, but thereafter the aortas of



Text-figure 3.

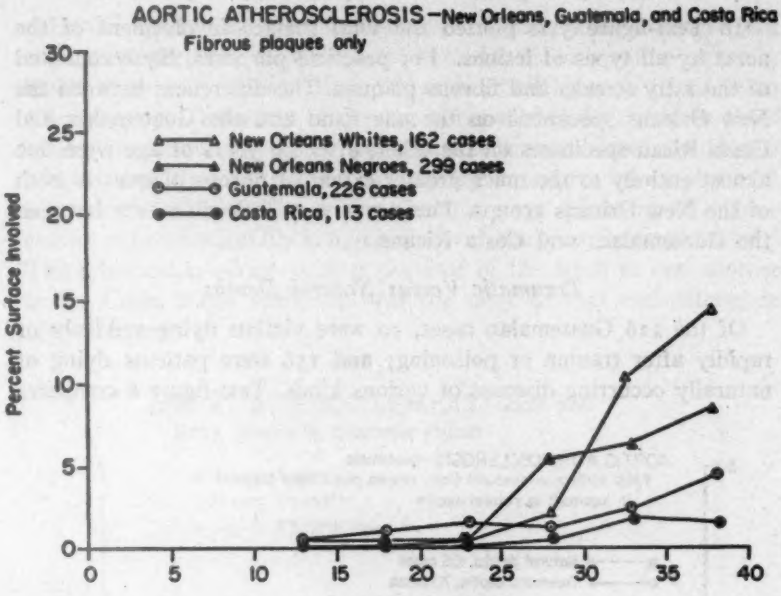
female patients were more severely affected by fatty streaks than those of the male. Many more specimens must be examined before these trends can be regarded as significant.

Fibrous Plaques

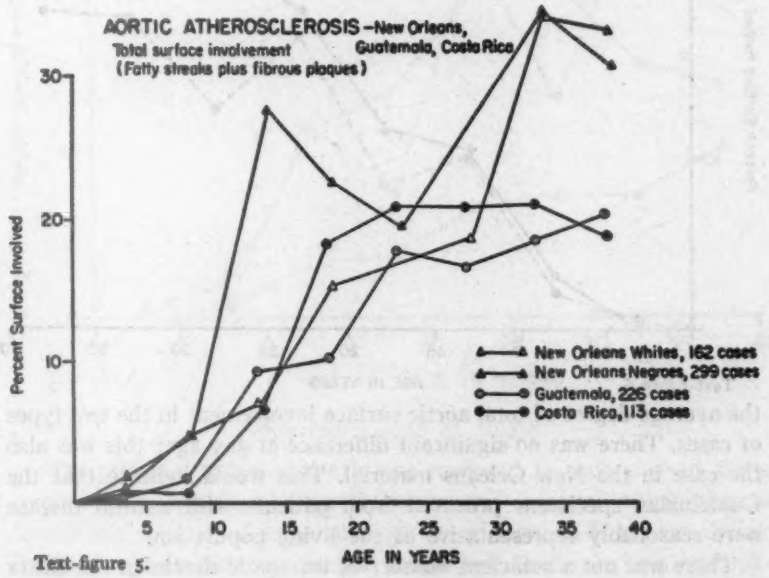
The average percentage of aortic surface covered by fibrous plaques is plotted for each region in Text-figure 4. Both Guatemalans and Costa Ricans had significantly fewer fibrous plaques by 40 years of age than either New Orleans whites or Negroes. There were no consistent differences between the two sexes in either Guatemala or Costa Rica with respect to fibrous plaques.

"Complicated Lesions"

Advanced lesions with ulceration, thrombosis, and calcification were so rare in the specimens from all 3 countries that the amount of surface area covered by them was insignificant.


AORTIC ATHEROSCLEROSIS—New Orleans, Guatemala, and Costa Rica
Fibrous plaques only
New Orleans Whites, 162 cases
New Orleans Negroes, 299 cases
Guatemala, 226 cases
Costa Rica, 113 cases
Percent Surface Involved
AGE IN YEARS

Text-figure 4.


AORTIC ATHEROSCLEROSIS—New Orleans, Guatemala, Costa Rica
Total surface involvement
(Fatty streaks plus fibrous plaques)
New Orleans Whites, 162 cases
New Orleans Negroes, 299 cases
Guatemala, 226 cases
Costa Rica, 113 cases
Percent Surface Involved
AGE IN YEARS

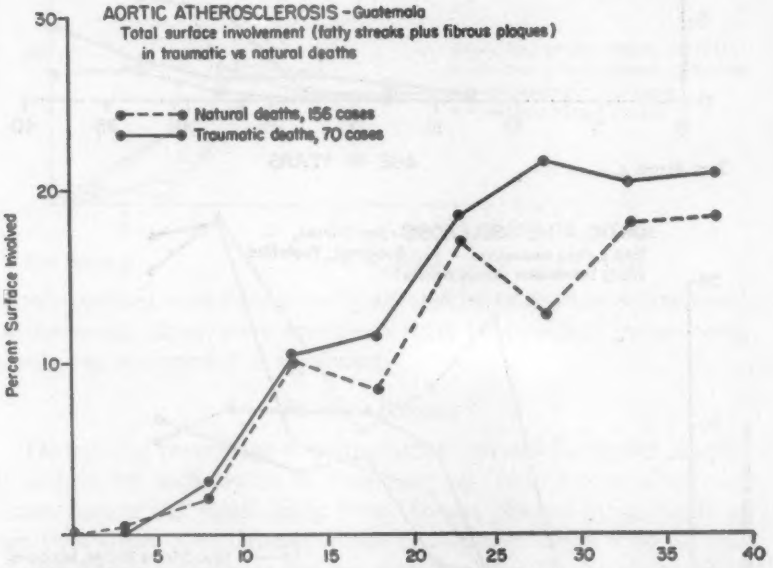
Text-figure 5.

Total Surface Area Involved by All Types of Lesions

In Text-figure 5 is plotted the total surface involvement of the aorta by all types of lesions. For practical purposes, these consisted of the fatty streaks and fibrous plaques. The differences between the New Orleans specimens on the one hand and the Guatemalan and Costa Rican specimens on the other, after 30 years of age were due almost entirely to the much greater extent of fibrous plaques in both of the New Orleans groups. There was very little difference between the Guatemalans and Costa Ricans.

Traumatic Versus Natural Deaths

Of the 226 Guatemalan cases, 70 were victims dying suddenly or rapidly after trauma or poisoning; and 156 were patients dying of naturally occurring diseases of various kinds. Text-figure 6 compares



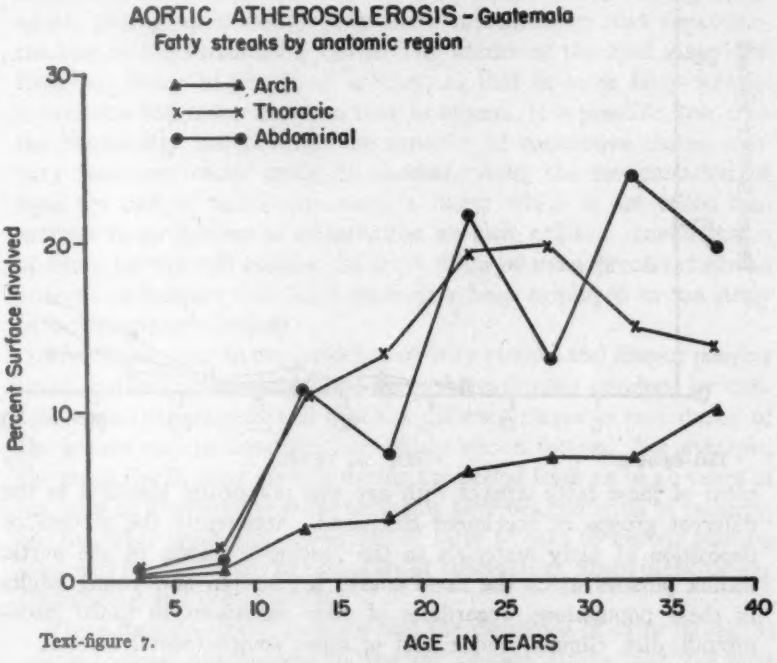
Text-figure 6.

the average degree of total aortic surface involvement in the two types of cases. There was no significant difference at any age; this was also the case in the New Orleans material. This would indicate that the Guatemalan specimens procured from patients with natural disease were reasonably representative of the living population.

There was not a sufficient number of traumatic deaths in the Costa Rican cases to make such a comparison.

Topographic Relationships

In the specimens from Guatemala, the descending thoracic and abdominal portions of the aorta were consistently more severely involved by fatty streaks than the arch at all ages up to 40 years (Text-fig. 7). The aortic rings were not available for study in these cases. The abdominal aorta was more severely affected by fibrous plaques, but there was some overlapping in degree of involvement between the arch and descending thoracic aorta (Text-fig. 8). These results were substantially in agreement with the New Orleans studies.[16] The relationship of the various portions of the aorta to one another in the Costa Rican specimens was the same as that encountered in New Orleans.



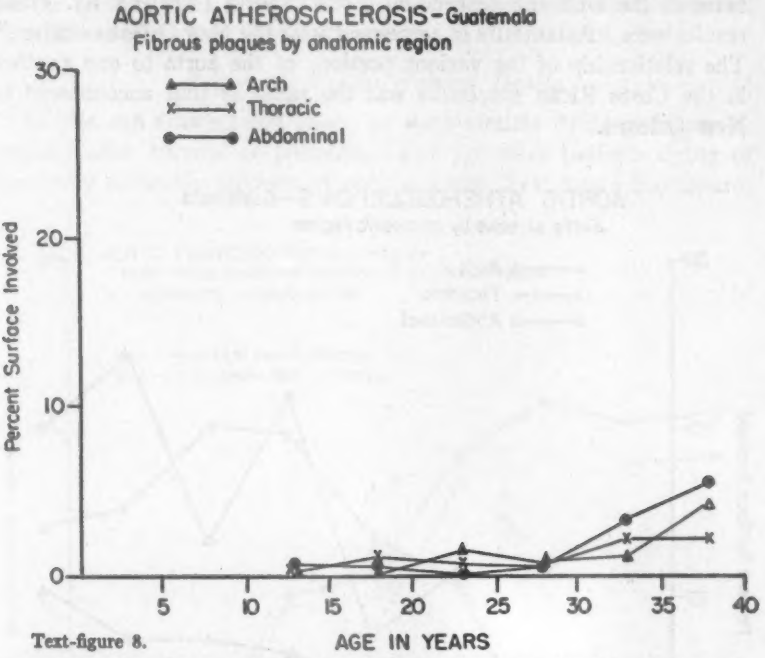
Text-figure 7.

Principal Cause of Death

The principal cause of death was reviewed in each case of the 4 groups to determine whether there was any unequal distribution of conditions known to have unusual association with atherosclerotic lesions, such as diabetes, familial hypercholesterolemia, and hypothyroidism. None was found.

Discussion

In view of the generally accepted belief that disease related to atherosclerosis is much less common in Guatemala and Costa Rica than in the United States, it came as a distinct surprise to us to find that all 3 population groups developed fatty streaks in the aorta at approximately the same rate. Furthermore, the pattern of develop-



Text-figure 8.

ment of these fatty streaks with age was practically identical in the different groups of specimens compared. Apparently the process of deposition of fatty materials in the mucinous matrix of the aortic intima pursues much the same course in children and young adults in these populations, regardless of wide variations in racial background, diet, climate, and a host of other environmental factors.

It also came as a surprise, after encountering the similarity in the extent of distribution of fatty streaks, to find that among Central Americans fibrous plaques were much less extensive than among New Orleanians. This may be a highly significant observation with respect to the nature of the process of atherosclerosis. It may also have an important bearing upon the difference in the incidence of clinical vascular disease.

Some may interpret these data to indicate that the fatty streaks have nothing to do with the development of fibrous plaques, since they show no parallel behavior when compared in this manner. This possibility must be considered until the relationship of the two types of lesions is established with more certainty. Such a conclusion is not supported by analysis of the data in other ways, however. For example, the topographic analyses show that fibrous plaques develop in the same anatomic areas as the fatty streaks, but appear some years later. Moreover, although difficult to document objectively, there exist numerous lesions of transitional type between simple fatty streaks and fibrous plaques.

Other possible interpretations may serve as the basis for future investigations into the causes and pathogenesis of atherosclerosis. It is conceivable that the lipid in the lesions may act as an injurious agent, setting up a slowly progressive inflammatory and reparative reaction in the surrounding tissue. The nature of the lipid may vary from one group of people to another, so that in some fatty streaks it excites a less severe reaction than in others. It is possible, too, that the reactability and proliferative capacity of connective tissues may vary from one racial group to another. Also, the concentration of lipid per unit of tissue may vary, a factor which is not taken into account in the system of quantitation we have utilized. Investigation of these factors will require the application of more precise chemical analytic techniques than have heretofore been employed in the study of atherosclerotic lesions.

The discrepancy in the incidence of fatty streaks and fibrous plaques noted emphasizes the likelihood that atherosclerosis develops by definite sequential stages[16] and that the different stages in maturation of the lesions may be controlled by widely varied factors. For example, the rapid rise in fatty streaks during the period from 10 to 20 years of age may be attributable to the changing hormonal patterns of puberty. On the other hand, the conversion of fatty streaks to fibrous plaques may well be related to the processes of coagulation, fibrin incrustation, thrombosis, and organization.

These data do clearly indicate that the formation of fatty streaks is not influenced significantly by the fat content of the diet, for it is established that both Guatemalan and Costa Rican individuals of the classes represented here consume much less fat in the diet than do North Americans.[10] The possibility remains that diet influences the formation of fibrous plaques, but a host of other factors must also be considered. Information concerning the state of nutrition in these patients is not sufficiently documented for statistical analysis, but it

should be pointed out that malnutrition is not incompatible with the existence of fatty streaks in the aorta. Many of the patients from Guatemala and Costa Rica suffered from malnutrition, even of advanced nature, but their aortas nevertheless revealed definite fatty streaks.

It is most interesting to note that the Costa Ricans, of almost exclusively white stock, developed lesions substantially of the same degree as occurred among the Guatemalans, who were of predominantly Mayan Indian stock. This indicates that an innate racial characteristic is not the only factor in the occurrence of different degrees of atherosclerosis, as was suggested by our earlier analyses of the New Orleans white and Negro races.[16]

It is encouraging, in anticipation of future efforts in the field of the geographic pathology of atherosclerosis, to find that grading the same material by independent investigators, using methods of quantitation similar in principle but different in details, has yielded results which confirm one another. Tejada and Gore[9] found that the severity of fibrotic, ulcerated, and calcified aortic lesions was much higher in the New Orleans specimens than in those from Guatemala at equivalent ages. Implied but not specifically stressed in their report was the similarity in the behavior of the fatty streaks in the two areas.

It is also of interest to note that among the cases of traumatic death in Guatemala there was no significant difference in the nature of the lesions from those encountered in instances of natural death. This was also the case in the New Orleans investigation. One would not expect this to hold true in the older age groups, where a significantly large proportion of the natural deaths occur as the result of atherosclerosis itself. Great caution should be exercised in making comparisons of this type in older age groups. For this reason, the collection of specimens from cases of traumatic death is highly desirable.

Summary

A quantitative comparison between the extent of various types of atherosclerotic lesions has been made in the aortas procured from a large number of individuals between 1 and 40 years of age in New Orleans, Guatemala and Costa Rica. Despite wide differences in racial and environmental backgrounds, these individuals show a similar pattern of development of fatty streaks in the aorta and a rapid rise in extent of involvement during the second decade of life. The most significant difference found was in the occurrence of fibrous plaques. These were far less numerous in the specimens from Guatemala and

Costa Rica than in those from New Orleans. This numerical difference appears to be correlated with a recognized variation in the incidence of clinical manifestations of atherosclerosis, and suggests that attention should be focused on factors influencing the conversion of fatty streaks to fibrous plaques. Diet appears to be relatively unimportant in the genesis of fatty streaks but may be of significance in the formation of fibrous plaques. The results obtained in this investigation should encourage expansion of similar quantitative and comparative studies in the geographic characteristics of atherosclerotic lesions.

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We are greatly indebted to Dr. Rodolfo Cespedes, Pathologist at the San Juan de Dios Hospital, San José, Costa Rica, for collecting anatomic specimens and supplying clinical and anatomic data.

HYPERPLASTIC INFANTILE CARDIOMEGALY

A Form of "Idiopathic Hypertrophy" With or Without Endocardial Fibroelastosis; and a Comment on Cardiac "Atrophy" *

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Idiopathic infantile cardiac hypertrophy may be defined as infantile cardiomegaly in the absence of detectable intra- or extra-cardiac lesions or abnormal circulatory dynamics capable of causing cardiac enlargement.

Weinberg and Himmelfarb,[1] impressed, as were others, by the striking change in the endocardium of more than half of these cases, devised the term "endocardial fibroelastosis." They believed that the stiffened endocardium was the cause of the cardiac "hypertrophy" and suggested for it a congenital and anomalous development. Since this offered both an etiology and a pathogenesis for more than one half of the cases, the term "idiopathic cardiac hypertrophy" was in large measure replaced by "endocardial fibroelastosis." The former term has been retained, but only for instances of cardiomegaly with normal endocardium.[2] In a preceding paper[3] it was suggested that idiopathic endocardial fibroelastosis, far from causing cardiomegaly, is a result of cardiac dilatation. In addition, the sclerotic endocardium is considered a support of, rather than an impediment to, myocardial function. If this be true, the etiology of infantile cardiomegaly with endocardial fibroelastosis again becomes, as "idiopathic infantile cardiac hypertrophy" has remained, a subject for investigation.

The term "idiopathic cardiac hypertrophy" indicates how firmly entrenched is the concept that any significant increase of cardiac mass beyond normal is a result of hypertrophy. This was not always so, and even today this interpretation is occasionally challenged.[4-7] Prior to the publication of Hepp's thesis in 1853,[8] wherein he suggested that hypertrophy was the mechanism of cardiac growth, cardiomegaly and normal postnatal heart growth were thought to reflect myocardial hyperplasia alone, or hyperplasia and some hypertrophy. As late as 1875 Zielonko,[9] on the basis of experiments with frogs and rabbits and

* This study was supported in part by the John R. Stark Memorial Research Fund.

Presented, in part, at the fifty-fifth Annual Meeting of the American Association of Pathologists and Bacteriologists, Cleveland, Ohio, April 25, 1958.

Received for publication, December 19, 1957.

study of the human heart, concluded that hypertrophy itself could not provide the entire explanation for cardiomegaly. In 1886 Goldenberg[10] reviewed the literature and reported his studies, concluding that hypertrophy was largely responsible for normal heart growth as well as for cardiomegaly. In 1889 Tangl,[11] studying the results of experimentally produced aortic stenosis in rabbits, decided that hypertrophy alone was the responsible mechanism for both cardiomegaly and normal growth. In 1911 Wideröe[12] published his conclusion that the heart of man enlarges by hypertrophy and not hyperplasia. A review of this subject is to be found in Cameron's book.[13]

The weight of accumulated evidence indicates that cardiomegaly is the result of hypertrophy. Therefore, when confronted with an enlarged infantile heart, the thought that it must represent cardiac hypertrophy seems not only reasonable but is almost automatic; yet, insofar as infants are concerned, there is no convincing direct evidence to support this conclusion.

Two problems have thus been posed; one concerns the etiology of infantile cardiomegaly, and the other its pathogenesis. Since elucidation of either of these may illuminate the other, the following investigation was undertaken.

Material and Method

Histologic preparations from the hearts of 58 individuals (49 infants and children and 9 adults) were examined. All specimens had been fixed in Zenker's fluid, embedded in paraffin, sliced at 6 μ and stained with hematoxylin and eosin. Tissue samples were procured from the convex lateral margin of the left ventricle, midway between base and apex. (When available, completed preparations were drawn from the slide files; when not, they were made from the previously prepared paraffin blocks.)

Thirty-two of the hearts were from presumably normal infants and children (Table I) and ranged in weight from 32 to 230 gm. In all of these, death was the result of an accident or acute infectious disease with neither clinical nor pathologic evidence of heart involvement arising from the fatal condition. Five hearts (Table II) were examples of idiopathic infantile cardiomegaly with endocardial fibroelastosis. Eight specimens (Table III), as will be demonstrated, were hypertrophic hearts. The cause for the hypertrophy of 3 was not evident; the others were from 5 children with chronic renal disease and hypertension or chronic valvular heart disease. The hearts of 4 cachectic children (Table IV) were considered to be "atrophic," as were those of 9 adults (Table V). All of the adult hearts fell within the weight range of the normal children's hearts.

TABLE I

Normal Hearts

Case no.	Age	Sex	Body wt. (kg.)	Length (cm.)	Heart wt. (gm.)	Nuclear density	Final diagnosis
C45-27	6½ mos.	F	6.9	64	32	51	Virus (?) pneumonia
C45-74	9 mos.	F	5.2	66	35	43.6	Ulcerative colitis
C45-59	7 mos.	M	5.9	68	35	43.6	(?)
C45-51	7 mos.	M	5.6	65	40	50.8	Volvulus and peritonitis
C54-51	17 mos.	M	8.8	74	49	48.8	Chronic meningitis
C45-96	18 mos.	M	8.1	77	50	41.6	Tuberculous meningitis
C54-110	13 mos.	M			52	43	Acute enteritis
C45-33	9 mos.	M	7.8	68	55	48.8	Acute meningitis
C45-68	2⅛ yrs.	M	9.4	84	66	34.8	Hydronephrosis and acute pyelonephritis
C53-41	3 yrs.	M	12.31	95	75	29.2	Acute tracheobronchitis
C51-38	2⅛ yrs.	M	14*	90	80	25	Burns
N50-536	3 yrs.	F	18.16		80	19.5	Sicklemia
C54-79	3 yrs.	F	13.16	96	85	18.8	Diabetes
N49-540	4⅛ yrs.	F			95	19.8	Poliomyelitis
C51-8	6 yrs.	F			95	25	Phlegmonous epiglottitis
C51-60	6 yrs.	M	14.25	105	97	22.8	Cystic pancreatic fibrosis
N49-70	5 yrs.	F			100	20.2	Meningococcemia
N50-92	5 yrs.	M	18.16*		105	21.2	Meningococcemia
N51-600	6 yrs.	F	20.4	106	105	19.4	Measles encephalitis
N53-293	6 yrs.	M	24.97*		115	21.6	Varicella encephalitis
C48-36	7 yrs.	F			120	15.6	Ruptured appendix
5868	7 yrs.	M			130	15.8	Subdural hematoma
C54-50	8-9/12 yrs.	M			148	15	Intracerebral aneurysm
6033	9 yrs.	M			150	18.8	Trauma
5762	6 yrs.	M			160	12.8	Post-measles (?) encephalitis
C48-19	12 yrs.	M	36.3	157	160	20	Acute leukemia
C52-89	8 yrs.	M	25.65	128	170	16.2	Acute and subacute hepatitis
N49-92	13 yrs.	M		149	180	10.8	Rubeola encephalitis
5809	12 yrs.	M			200	10.4	Subdural hematoma
N52-549	15 yrs.	F	45.4*	157	230	10.4	Viral encephalitis
N52-513	11 yrs.	M	50*		250	9	Trauma
C46-60	12 yrs.	F	46.7	152	250	7.6	Acute hepatitis

* Estimated weight.

TABLE II
Hyperplastic Hearts

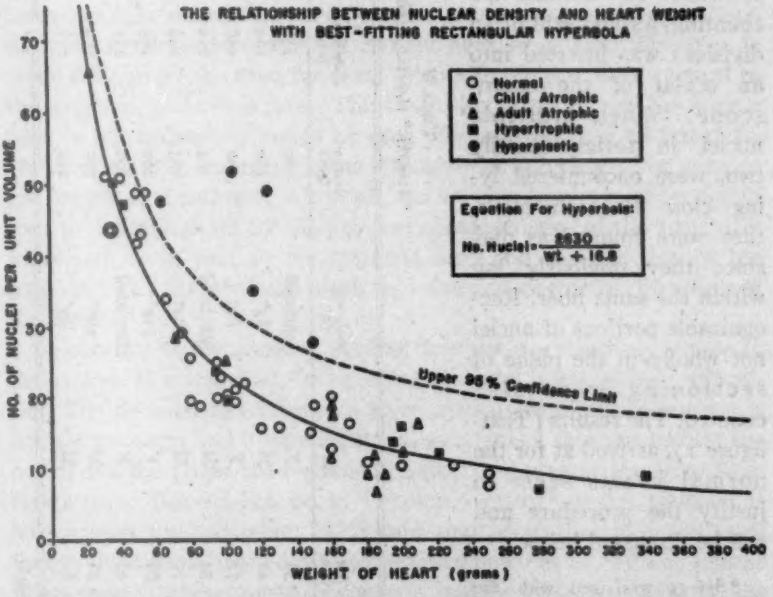
Case no.	Age (mos.)	Sex	Body wt. (gm.)	Heart wt. (gm.)	Expected heart wt. by age (gm.)	Nuclear density	Estimated normal no. of nuclei for mass	Estimated no. nuclei
C52-43	4½	M	5,217	64	28	47.6	34	1.4
N50-104	5	F	4,760	105	29	51.8	21	2.4
S56-55	3	M	4,680	116	23	35	19	1.8
C52-90	4	M	6,846	125	27	45.4	17.6	2.5
5730	7½	M	6,050	152	35.5	27.6	14.5	1.9

TABLE III
Hypertrophic Hearts

Case no.	Age	Sex	Body wt. (kg.)	Heart wt. (gm.)	Expected heart wt. by age	Nuclear density	Final diagnosis
C45-8	3 mos.	M	6.8	37	23	50.4	Acute meningitis
C54-33	2½ mos.	F	3.7	43	23	47	Subdural hematoma
N50-556	20 mos.	F	9.5	95	56	23.4	Mucopurulent bronchial plugs
C49-56	6 yrs.	M	14.5	195	94	13.8	Chronic pyelonephritis
C48-42	5 yrs.	M	15.0	200	85	16.2	Chronic glomerulonephritis
C54-81	3 yrs.	F	13.3	221	59	12.2	Mitral insufficiency with endocardial sclerosis
C54-89	8 yrs.	F	23.6	278	110	7.2	Probably chronic rheumatic fever
N50-299	12 yrs.	F	36.6	340	124	8.6	Chronic rheumatic fever

TABLE IV
"Atrophic" Hearts: Children

Case no.	Age	Sex	Body wt. (kg.)	Heart wt. (gm.)	Expected heart wt. by age (gm.)	Nuclear density	Final diagnosis
C45-69	8 mo.	M	5.1	23	37	65.6	Aspiration of gastric contents
C53-41	8 yrs.	M	12.3	71	110	28.4	Anaplastic Ca liver
N46-289	13 yrs.	F	32.5	100	124+	19.9	Adenocarcinoma lung
C51-88	11 yrs.	M	28.0	100	122	25	Mesothelioma peritoneum



Text-figure 1. The course of the hyperbola (solid line) fitted to the data obtained from 32 normal hearts of infants and children describes normal cardiac growth. Only one point among the 32 sets of coordinates falls beyond the 95 per cent confidence limit. All points determined for the hypertrophic hearts of infants and children as well as those for the atrophic adult and children's hearts fall within this limit. All of the 5 hyperplastic enlarged hearts with endocardial fibroelastosis lie beyond the 95 per cent confidence limit and attest to the existence of two dissimilar populations.

All cases but 6* were collected from the Departments of Pathology of the Cincinnati General Hospital and the Children's Hospital and Research Foundation of Cincinnati. All sections were examined through the same microscope at a magnification of 900 times. The diameter of the field was 150 μ.

The myocardial nuclei of 5 fields in each specimen were counted and the arithmetical average taken as representative of the left ventricle. To facilitate the counting, a ruled grid of 16 divisions was inserted into an ocular of the microscope. When multiple nuclei in series, usually two, were encountered lying close to each other, they were counted as one, since they obviously lay within the same fiber. Recognizable portions of nuclei not wholly in the plane of sectioning were also counted. The results (Text-figure 1), arrived at for the normal hearts seem to justify the procedure and will be discussed below.

*S56-55 was used with the permission of Dr. F. P. Cleveland of the Coroner's Office of Cincinnati. Cases 5868, 6033, 5762, 5809, and 5730 were used with the kind permission of Dr. Wiley D. Forbus, Department of Pathology, Duke University School of Medicine, Durham, N.C.

TABLE V

"Atrophic" Hearts: Adult

Case no.	Age (yrs.)	Sex	Body wt. (lb.)	Heart wt. (gm.)	Body length (cm.)	Expected heart wt. by height*	Nuclear density	Final diagnosis
N50-566	37	F	emaciated	160	156	256 ± 30	18.8	Carcinoma, cervix
N49-583	42	M	85*	160	163	308 ± 40	11.8	Carcinoma, renal
N53-679	73	F	85*	180	156	256 ± 30	9.2	Carcinoma, renal
N46-278	34	M	emaciated	185	162	306 ± 40	6.6	Tuberculosis
N53-194	71	M	80*	185	146	275 ± 40	13	Carcinoma, colon
N53-344	62	F	120*	160	165	272 ± 30	17.4	Carcinoma, cervix
N46-251	47	F	emaciated	190	151	247 ± 30	9.4	Tuberculosis
N54-24	32	M	100*	200	164	310 ± 40	12.2	Carcinoma, bile ducts
N52-361	62	M	90*	210	172	325 ± 40	16.4	Carcinoma, stomach

* Estimated weight.

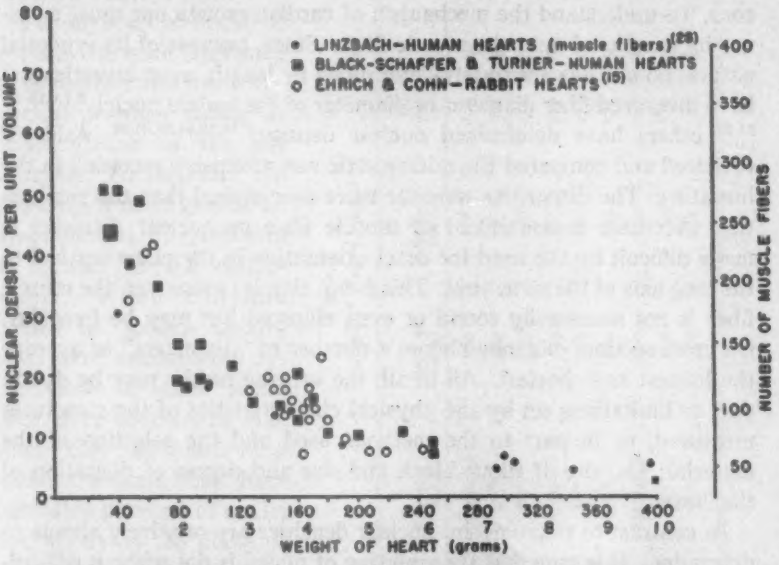
In the course of this work it was learned that relatively few of the heart preparations reviewed were useful. To qualify for inclusion in the series, the muscle fibers had to closely approximate each other with little or no interstitial space. Because of an obvious difference in the size of the nuclei, largest in the *trabeculae carneae*, only those nuclei were counted whose fibers made up the innermost layer of the compact myocardium adjacent to the *trabeculae carneae*. In addition, the muscle bundles had to be sectioned in their long axis. As a result of these criteria, not more than one of every 5 hearts whose sections were originally examined was found acceptable.

The choice of "nuclear density" (number of nuclei in a constant volume) as a measurement of growth was made for the following reasons. To understand the mechanism of cardiac growth one must measure its functional unit, the muscle fiber. Since, because of its syncytial nature, no one has accurately determined its length, most investigators have measured fiber diameter or diameter of the muscle nuclei;[6,7,9,10-12,14-23] others have determined nuclear density.[6,14,15,18,21,24-28] Ashley[29] reviewed and compared the micrometric measurements recorded in the literature. The disparities were far more pronounced than the similarities. Accurate measurement of muscle fiber or nuclear diameter is made difficult by the need for exact orientation in the plane vertical to the long axis of the structures. This is not simple; moreover, the muscle fiber is not necessarily round or even ellipsoid but may be irregular. On cross section, one may choose a number of "diameters" or average the longest and shortest. All in all, the varying results may be due in part to limitations set by the physical characteristics of the structures measured, or in part to the methods used and the selection of the material; i.e., site of tissue block and size and degree of dilatation of the heart.

In contrast to micrometry, nuclear densities are relatively simple to determine. It is true that the counting of nuclei is not without difficulties. The decision to recognize a given nucleus as a muscle nucleus is not always easy, and it may be stated as a truism that the smaller the nuclei and the larger the number in a field, the greater the error from this source. The application of "nuclear density" to our problem is based upon the following. It is said that after the third postnatal month the human heart ceases to produce new nuclei;[24,26] i.e., muscle fibers, and further growth becomes a matter of hypertrophy of the extant fibers. In such a system the relationship between the mass of the tissue and the number of units in a given volume is reciprocal. A curve which describes this relationship is the hyperbola, wherein the product of the two factors forming a locus is a constant anywhere on

the curve. A hyperbola of the form $y = \frac{k}{x + a}$ was fitted to the data of Table I by the method of least squares.[27] The fitted curve along with our data are shown in Text-figure 1 and appear to justify the assumption of a hyperbolic relation.

If this relatively simple means of measuring cardiac growth is accurate, data from independent sources should more or less produce our results. The only identical data which could be found were those of Ehrich and Cohn.[15] When the nuclear densities were plotted against the heart weights of their rabbits, a similar relation to that seen in our human hearts was noted. The data of Ehrich and Cohn are shown together with ours in Text-figure 2. Linzbach[28] plotted the weight of a

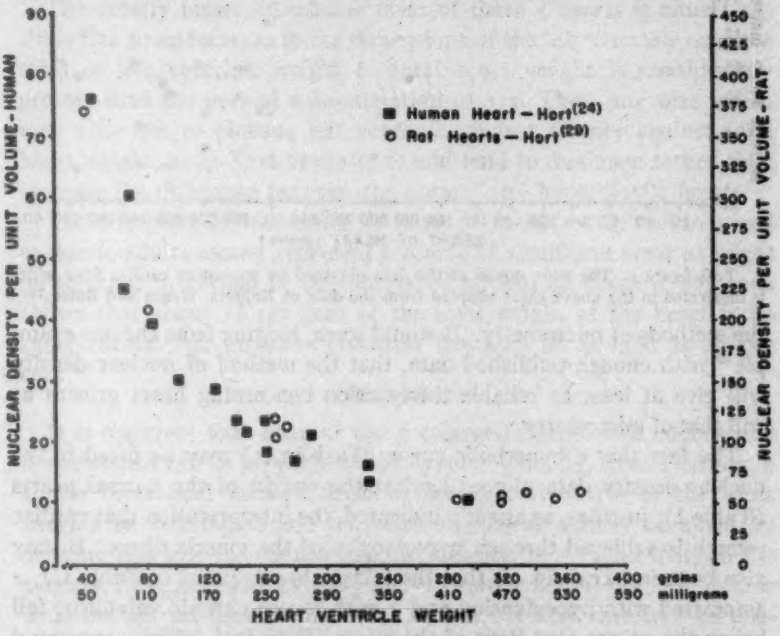


Text-figure 2. The figure illustrates the close similarity of 3 independently collected groups of data correlating normal heart muscle fiber and nuclear density with heart weight of both man and rabbit.

series of hearts against their muscle fiber numbers, but exact values were not published. These were therefore approximated from a graph and, as indicated in Text-figure 2, reveal a similar distribution to the hearts of our series and that of Ehrich and Cohn.[15] In both of the former, nuclei and not muscle fibers were counted. Hort[29] in 1951 published data on normal rat hearts correlating ventricular weight with nuclear density and in 1953[24] similar data on normal human hearts. When his data are plotted (Text-fig. 3) as above, the close similarity of the two groups is at once demonstrated.

These illustrations of the reproduction of our results by independent investigators, insofar as normal heart growth is concerned, demonstrate that in at least 3 mammals, man, rabbit and rat, heart growth progresses in the same manner and may be reasonably well described by means of "nuclear density."

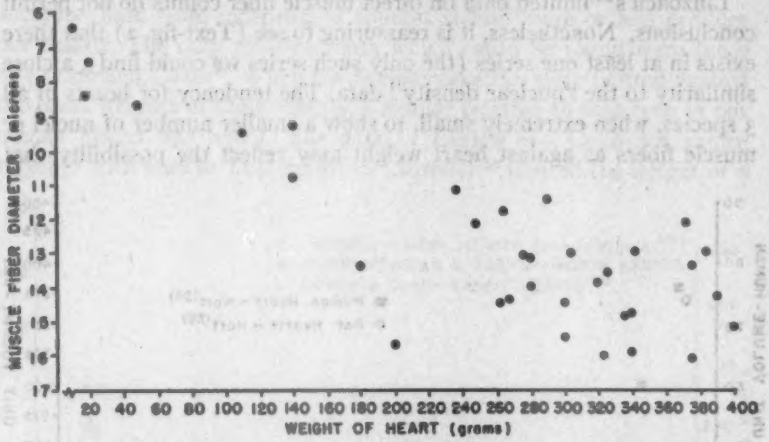
Linzbach's[28] limited data on direct muscle fiber counts do not permit conclusions. Nonetheless, it is reassuring to see (Text-fig. 2) that there exists in at least one series (the only such series we could find), a close similarity to the "nuclear density" data. The tendency for hearts of all 3 species, when extremely small, to show a smaller number of nuclei or muscle fibers as against heart weight may reflect the possibility that



Text-figure 3. The striking similarity of the distribution of the points relating cardiac nuclear density and heart weight of man and rat resembles that of man and rabbit in Text-figure 2.

these small hearts have not yet produced their definitive number of muscle fibers.[24,26] Roberts, Wearn and Boten[19] measured and averaged the width, through the nucleus, of muscle fibers of left and right ventricles of a series of human hearts. These varied in weight from 13 to 400 gm. While the fibers showed an increase in width in the heavier hearts, the spread, as is readily seen in Text-figure 4, is considerably greater than that of the "nuclear density" series.

It seemed desirable to write at such length in justification of our method since, with only a few exceptions,[5,6,15,24,26,29] most authors have found it advantageous to place their trust in the incomparably more difficult methods of micrometry. There is no available evidence objectively demonstrating the degree of accuracy or reproducibility of



Text-figure 4. The wide spread of the data obtained by measuring cardiac fiber width is illustrated in the above chart adapted from the data of Roberts, Wearn and Boten.[19]

the methods of micrometry. It would seem, judging from the one example[19] with enough published data, that the method of nuclear density will give at least as reliable information concerning heart growth as will that of micrometry.

The fact that a hyperbolic curve (Text-fig. 1) may be fitted to the nuclear density data plotted against the weight of the normal hearts (Table I), justifies, as already indicated, the interpretation that cardiac growth is achieved through hypertrophy of the muscle fibers. It may also be seen (Text-fig. 1) that the 8 large hearts listed in Table III, 2 associated with hypertension and 3 with severe chronic valvulitis, fall below the 95 per cent limit of the curve. Their loci, which correspond to those of normal hearts of similar weight, is good evidence that hypertrophy is, in effect, an acceleration of normal growth. This is not unexpected since the weight of the left ventricle normally is *circa* 73 per cent of the combined weight of the two ventricles, and about 50 per cent of the total heart weight.[22,30-33] Therefore, the dimensions of its fibers would reflect the total heart mass more closely than those of the other chambers.

While hypertrophic cardiomegaly is an extension of normal growth disproportionate to the individual, "atrophy" is a reversal of normal

growth proportional to the individual. This becomes evident from the positions occupied by the hearts of Tables IV and V. These hearts were taken from cachectic children (Table IV) and cachectic adults (Table V). Their loci in Text-figure 1 are again all within the 95 per cent confidence limit. "Atrophy" of the heart is more fully discussed in the terminal section of this paper.

In contrast to the 53 normal, hypertrophic, or "atrophic" hearts, the 5 large hearts with endocardial fibroelastosis (Table II) assume, in Text-figure 1, a unique and significant position. They all fall beyond the 95 per cent limit, indicating that the hypothesis that these hearts belong to the same population as the others is untenable.

The greatly increased cardiac mass of these 5 hearts is almost entirely due to an increase in the dimensions of the left ventricle, and the ratio of left ventricle weight to total heart weight is considerably greater than the normal approximation of 1:2. Thus, any bias which may arise out of plotting left ventricle nuclear density against total heart weight, as in Text-figure 1, should tend to minimize rather than increase the difference between the normal and hyperplastic hearts.

The subepicardial fat which is minimal to absent in children and cachectic adults cannot represent a source of significant error as might be the case in normal or overnourished adults. In these it has been shown that about 16 per cent of the total weight of the heart is its subepicardial fat. In some instances this may be as high as 23 per cent.[24]

Discussion

It is apparent that none of the 5 enlarged hearts with endocardial fibroelastosis can be properly called hypertrophic. If, from Text-figure 1, the theoretical nuclear densities for normal hearts of the same weights be determined and an index of nuclear density obtained by dividing this into the counted nuclear density, it is found that, on the average, there are twice as many nuclei (muscle fibers) per unit volume as in the normal heart of the same weight. It may therefore be concluded that the muscle fibers are smaller than those of the normal heart and approximately twice as plentiful. This is true hyperplasia.

The cardiac fibers, in normal development, have been calculated to pass through approximately 35 generations, assuming origin from a single cell.[22] The last division, believed by some to be amitotic,[6,15,22] occurs sometime between birth and 3 months of age. If there should be one supranormal generation, it would result in a doubling of the number of units. This may have occurred in our cases.

Since the oldest of the 5 patients was only 7½ months and the index of nuclear density here was 1.9 (Table II) it appears permissible to

suggest that, as in the normal heart, even in these relatively huge hearts, no further nuclear divisions occurred beyond the first few postnatal months. It is highly probable that the extra generation may have come into being *in utero*. This is supported by Race and Black-Schaffer's[5] identification of myocardial hyperplasia in a premature newborn of 6¼ months gestation.

The demonstration of a possible supernumerary generation of heart cells immediately raises the question, "What may be the functional and morphologic consequences of *in utero* cardiac hyperplasia?" During late fetal life the systemic blood pressure is low, and the left and right ventricles share the circulatory burden in almost equal measure.[22] The demand placed upon the myocardium is small. After birth the shift of the circulatory burden to the left heart drastically alters this situation which is reflected by the changing mass of the two ventricles. At birth the relation of free* right ventricular to total ventricular muscle is 38.6 per cent to 61.3 per cent[22] or 29.7 per cent to 70.3 per cent.[21] By the end of the first postnatal month, the right ventricle has lost approximately 20 per cent of its original weight, and while the heart has not ceased its remodeling, for practical purposes it may be said to have achieved its definitive form. In this form the left ventricle does the major part of cardiac work.

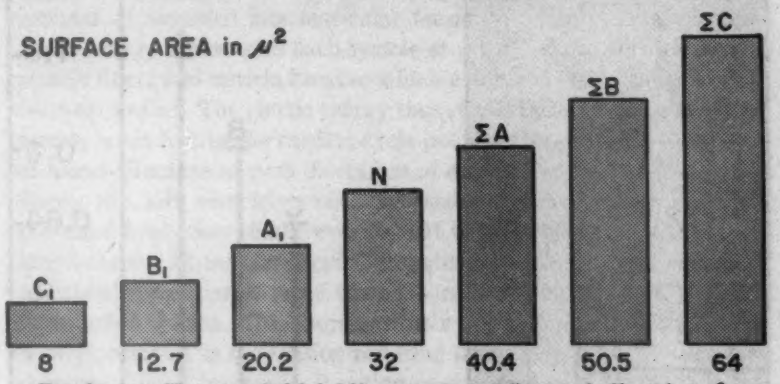
If it is assumed that before birth the hyperplastic heart was composed of twice the normal number of fibers but retained normal gross dimensions,[5] it follows that the myocardium was adequate to its load. This conclusion rests upon the proposition that the heart is an organ whose structure and organization are largely determined by the work it does[35,36] and the dynamics of the blood stream.[37] For example, anomalies, such as *in utero* closure of the *foramen ovale*, initiate a series of changes resulting in very marked alterations of cardiac structure and appearance.[3,38] Patten states,[37] "It is well known that an excised chick heart kept alive by tissue culture methods spends its energies in beating and, to a less extent, in unorganized growth of its tissue elements. It does not, as do many other explanted parts of the growing body, differentiate morphologically under the influence of its 'inherent developmental potencies.' This would naturally lead one to turn inquiringly toward the possible moulding influences exerted by the hydrodynamic effects of blood currents and eddies." In this respect Rodbard,[39] by means of models, has demonstrated the development of

* The free right ventricular muscle is the myocardium cut away from the interventricular septum along its junction and dissected from its annulus fibrosus. The interventricular septum is considered a part of the left ventricle.

valve cushions, valves, and structures analagous to anomalies, in response to a stream of water.

It would seem reasonable, therefore, to assume that a heart which has normal dimensions and gross structure for a given individual, is functionally adequate. With the changing hemodynamics of the postnatal era, however, the hyperplastic myocardium becomes inadequate. This is reflected by the progressive cardiac dilatation and cardiomegaly.

It has already been pointed out earlier that a doubling of the number of nuclei (muscle fibers) may be the result of one supernumerary generation. One wonders why 2 or even 3 such generations have not been discovered. A reasonable answer is found in a consideration of fiber surface area, length, and volume accompanying the volumetric substi-



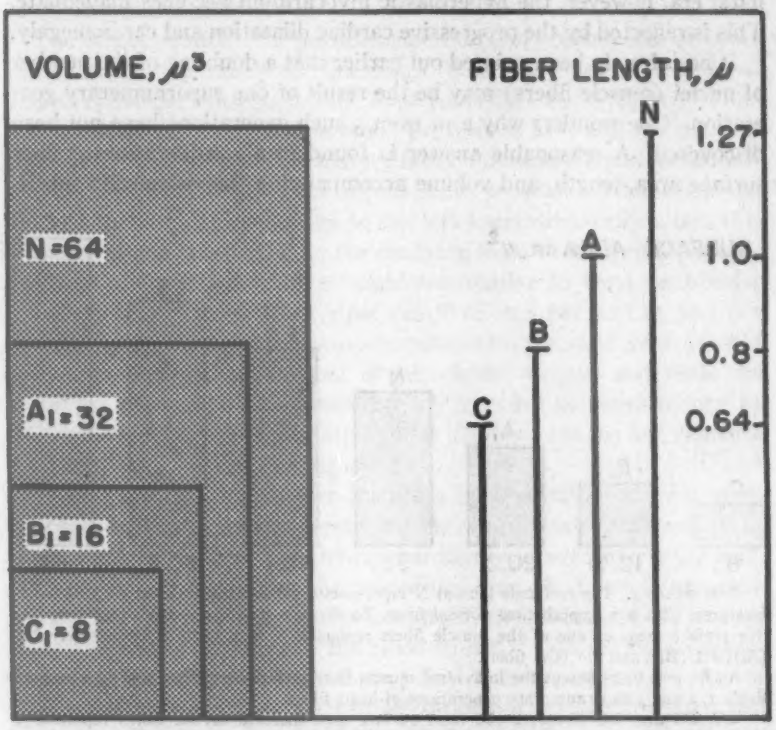
Text-figure 5. The rectangle labeled N represents a cardiac muscle fiber of 32 μ^2 surface area. This is a hypothetical normal fiber. To the left of N are ordered the comparative surface areas of one of the muscle fibers replacing N in a ratio of respectively 2:1 (A_1), 4:1 (B_1), and 8:1 (C_1) fibers.

A_1, B_1, and C_1 represent the individual muscle fiber surface area when there are respectively 1, 2 and 3 supernumerary generations of heart fibers.

ΣA, ΣB and ΣC illustrate the total surface area increase of the fibers replacing 1 normal fiber in the same circumstances.

tution of one by two or more fibers. Text-figures 5 and 6 illustrate the relationships of each of the types of hyperplastic fibers which would be found in hearts with respectively 1 (A), 2 (B), and 3 (C) supernumerary cell generations as compared to a hypothetical normal fiber (N). This "normal" fiber, the "equivalent body" of Linzbach,[6] serves admirably the geometric analysis of heart fiber structure. It is a cylindrical disk whose height (length) is always its radius divided by π ($h = \frac{r}{\pi}$). Hence: Volume $= r^3$ and Surface Area $= 2r^3$. As the normal postnatal heart grows, since its basic shape is retained, and the number of fibers is constant, it may be assumed that the

individual fiber proportions also remain constant.* If one substitutes 2 fibers (A) for 1 (N), volumetrically (Text-fig. 6), then each of the 2 (A) obviously contains 50 per cent of the original volume. Since the external proportions of the original and the new fibers are identical, a simple calculation, employing Linzbach's "equivalent body" formula,



Text-figure 6. As in Text-figure 5 the rectangle N represents the same hypothetical normal muscle fiber with a volume of 64 μ^3. A_1, B_1, and C_1 within N show the proportional decrease of individual cell volume (contractile substance) when there are 1, 2 and 3 supernumerary heart muscle generations. To the left, the vertical lines show the progressive decrease in the resting length of the fibers in the same circumstances.

reveals that the combined surface area (Text-fig. 5) of the 2 smaller fibers (A) is 26.2 per cent greater than that of the original fiber (N). In addition, it is demonstrated that each of the 2 fibers is 21 per cent shorter than the original (Text-fig. 6).

When one recalls that the energy of isometric contraction of a muscle fiber is a function of its diastolic length, it becomes evident that a

* This statement of "constant proportionality of normal cardiac dimensions" will be the subject of a separate study.

hyperplastic heart with a 2:1 muscle fiber substitution may be, *a priori*, composed of weaker fibers. While it might seem that the larger number of fibers might compensate for their individual weakness, this is not the case. When it is considered that the efficiency of the mammalian heart has been variously estimated to be from 10 to 20 per cent,[36] it is obvious that the permanent diversion of even a relatively small amount of energy from useful work may seriously reduce myocardial effectiveness.

It is proposed that the increased surface area of the hyperplastic muscle fibers in conjunction with their reduction of contractile substance contribute in several ways to such a diversion of energy. One may be by an increase of "friction" (internal work). Another is by an increase of so-called interfascicular tension.[36] This has been defined as the energy stored with each systole at points of attachment between muscle fibers and muscle bundles which contract in different directions from each other. The elastic energy thus stored by stretch of the attachments, is not lost to the cardiac cycle but diverted from the movement of blood. Because of such diversions of energy and the shortness of its fibers, the left ventricle, when subjected to the increasing normal postnatal load, may dilate well beyond the normal range. While this lengthens the fibers and should strengthen systole, it is associated, if sufficiently great, with more than a corresponding increase of lateral myocardial tension. This increase, for a sphere, may be expressed as $T \propto r^2$, where T is the tension resulting from the action of hydrostatic pressure (blood pressure) P acting over a radius of r.[3,40] It is suggested, therefore, that the increased tension may more than offset any advantage accruing to the congenitally weaker fibers through their stretching. In addition, it is conceivable that the reduced volume of contractile substance in the small fibers results in a contraction of the physical limits over which Starling's law operates. Thus, instead of increasing stroke force the dilatation may still further reduce the efficiency of the hyperplastic fibers. It is, perhaps, this excessive dilatation or stretch which initiates the growth or "hypertrophy" which, stepwise,[6] leads to the cardiomegaly so characteristic of these hearts. The relatively enormous mass which the largest attain is made possible by the development of endocardial fibroelastosis which relieves the myocardium of part of the laterally acting tension; however, this metaplasia develops only after a considerable degree of dilation has been achieved.[8]

The explanation of the question, "Why were not 2 or 3 or more supernumerary generations of muscle fibers found?" may likewise be assayed after examination of Text-figures 5 and 6. If two supernum-

erary generations (B) were to develop, the total increase of muscle surface area (ΣB), would be 58.7 per cent beyond normal, the fibers would each be 37 per cent shorter than normal, and their volume only 25 per cent of normal. It is possible that a heart composed of such units could not maintain postnatal circulation, and the child would die shortly after birth, if not *in utero*. The results of 3 supernumerary generations (C) would be even more catastrophic since the total surface area (ΣC) would be 100 per cent greater than normal, the fibers shorter by 50 per cent and their volume only 12.5 per cent of normal. This would surely be a lethal, *in utero*, anomaly.

An alternative thesis must be considered which, while less probable, nonetheless deserves exploration. If one assumes, for these infantile hearts, as do some for adult myocardosis,[41] that the initial, abnormally great (postnatal) dilatation is the consequence of defective but dimensionally normal fibers, then cardiomegaly must be considered the result of true hypertrophy. This it is not, since the fibers are demonstrably small. Is it, however, possible that an intial true hypertrophy was changed to hyperplasia? It is said that hyperplasia develops in the adult heart when it achieves a mass of around 500 gm. (critical size).[6] While this phenomenon has been partly and independently confirmed in the adult, it has not been recorded for the infantile heart. It is, of course, conceivable that in the first year of life cardiac hypertrophy may be an even more potent stimulus to hyperplasia than in the adult.

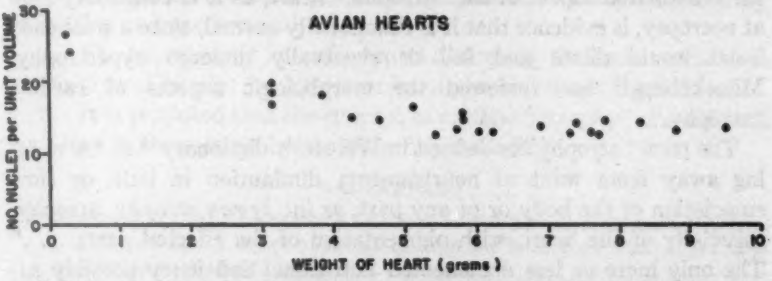
That this thesis is probably incorrect when applied to the infant, is indicated by several observations and considerations. The presence of true hypertrophy in a 2½ month infant of this series (Table III) and in a 4 month infant in the study of Race and Black-Schaffer,[5] shows that significant hypertrophy without hyperplasia may occur in early infancy. It must be added that neither of these hearts was as large, absolutely or relatively, as the smallest of the hyperplastic hearts. There is, however, the previously mentioned observation of an instance of cardiac hyperplasia in a heart of normal dimensions from a 6¼ month premature newborn.[5] This is certainly indicative of a prenatal change which may be the cause and not the result of cardiomegaly.

Hypertrophy was the mechanism of enlargement in the remaining cases of cardiomegaly of Table III. While these patients were not infants but children, ranging from 3 to 12 years of age, the degree of cardiomegaly of at least one (C 54-81), a 3 year old, is equal to that of the hyperplastic hearts; nonetheless, it was hypertrophic. This hypertrophic organ indicates that even at so early an age "critical size" must be more than 221 gm. It would seem that if the concept of "critical size" is absolute, no infantile heart could be affected, for no

infant could possibly produce nearly so large a heart (500 gm.), and still have space for respiration.

It has been demonstrated[17,19,25] that at birth the ratio of capillaries to heart fibers in a given field is 5:1. As the heart fibers increase in size, the concentration of capillaries to myocardial fibers decreases, until in the normal adult the ratio is 1:1.6.[25] This has led to the suggestion[16,25,42] that, because of the greater increases of volume ($V \propto r^3$) as against surface area (S.A. $\propto r^2$) of the muscle fibers, and the increasing distance across which gases and other substances must diffuse from capillary to nucleus,[16] the hypertrophic heart may be less efficient than the normal. The Berlin school of Linzbach and his colleagues[6,25,26] utilized this concept to explain the advantages of amitotic fission (hyperplasia) of heart fibers which have reached a "critical size"; namely, a reduction in the volume to surface area ratio. Whatever the merits of these interpretations may be, they would not seem to apply to the infantile heart which, regardless of the degree of cardiomegaly, never approaches "critical size."

As already indicated MacMahon[4] described an example of "hyperplastic cardiomegaly" in a 6-month-old infant. This diagnosis was based upon the presence of unusual numbers of mitotic figures, sometimes as many as 3 or 4, in a single low-power field. No measurements or counts of any kind were reported. MacMahon described the presence of anomalous ureters, chronic renal disease and death in uremia. The heart, which weighed only 60 gm., could conceivably have been an example of hypertensive cardiomegaly. No blood pressure



Text-figure 7. Constructed from the data of Ehrich and Cohn[15] this figure illustrates that in the growing normal chicken heart there is relatively little change in nuclear density (size of myocardial fibers) over the entire range of cardiac growth.

determinations were mentioned. Race and Black-Schaffer[5] were convinced by their nuclear counts that some cases of infantile idiopathic cardiomegaly were associated with hyperplasia. They went so far as to suggest that hyperplastic cardiomegaly might be a congenital defect of development whereas hypertrophic cardiomegaly was acquired. They

offered no explanation for the pathogenesis of the cardiomegaly and were not concerned with endocardial fibroelastosis, either idiopathic or otherwise.

Cardiac hyperplasia is the physiologic means of heart growth in chickens.[15,24] Text-figure 7, constructed from the data of Ehrich and Cohn,[15] illustrates this basic difference between the mammalian and the avian heart. No information on the hearts of other avian representatives could be found in the literature consulted so that no generalizations may be made on this apparently divergent means of normal cardiac growth.

Hypocardia ("Cardiac Atrophy")

Not directly related to the preceding discussion, but a side product of the examination of the "atrophic" hearts and the position they occupy in Text-figure 1, is a comment on a concept which enjoys wide currency; namely, that the muscle fibers of "atrophic" hearts suffer regressive changes and a diminution of their numbers.[14] In the "atrophic" but functionally normal hearts of Tables IV and V, there was no histologic evidence of such changes.

The size of normal heart muscle fibers is directly related to the diastolic volume of the ventricles. In progressive cachexia the blood volume decreases. This is associated with a reduction in the size of the ventricles and the muscle fibers, but this does not relieve the individual fibers of their proportional share of work. In this sense, as long as the heart continues to beat, there can be no cardiac "atrophy." The diminutive undilated aspect of the "atrophic" heart, as it is commonly seen at necropsy, is evidence that it is functionally normal, since a weakened heart would dilate and fail or eventually undergo hypertrophy. Mönckeberg[43] has reviewed the morphologic aspects of cardiac "atrophy."

The term "atrophy" is defined in Webster's dictionary[44] as "A wasting away from want of nourishment; diminution in bulk or slow emaciation of the body or of any part, as in: *brown atrophy*, atrophy, especially of the heart, with pigmentation of the affected part; . . ." The only more or less documented nutritional deficiency possibly affecting the heart of man is beri-beri.[45] It is characterized by cardiomegaly and not "atrophy" despite the fact that, in the Occident, beriberi is usually associated with general malnutrition. There is no evidence to indicate that the smallness of an "atrophic" heart reflects a "wasting away" or functional inadequacy. Under these circumstances the above definitions of atrophy apply but poorly to the heart. The importance of circulatory dynamics in molding the heart cannot be overemphasized, yet the term "atrophy," as currently used, is, in

effect, a denial of that importance. While fully aware that cardiac "hypertrophy" is equally indefensible, the probabilities of effecting a change in its use are exceedingly remote. It is, however, reasonable to suggest the substitution of "hypocardia" for "atrophy."

Summary and Conclusions

1. A hyperbola was fitted to data obtained by plotting nuclear density against weight of 32 normal hearts. These varied from 32 to 250 gm. The hyperbola describes normal cardiac growth.

2. By this method the well established fact is confirmed that normal heart growth is a result of hypertrophy of extant muscle fibers.

3. Nuclear density determinations make possible the objective demonstration that cardiomegaly associated with hypertension or chronic valvular disease is an abnormal extension of the hypertrophy of normal growth.

4. The technique permits the demonstration that some cases of idiopathic infantile cardiomegaly are examples of true hyperplasia. It is proposed that this hyperplasia represents a congenital defect and not necessarily a response to abnormal postnatal hemodynamics.

5. It is suggested that the fiber sarcoplasm is probably, in and of itself, normal but that the abnormal physical relationships of volume of contractile substance, resting length, and surface area are such as to decrease the efficiency of the myocardium.

6. This decrease of efficiency suffices to present the rapidly increasing, but normal, left ventricular load of the first postnatal month as an unphysiologic burden to which the hyperplastic ventricle responds by dilatation and cardiomegaly.

7. The hyperplastic infantile hearts enlarge by the same mechanism as do normal hearts; by hypertrophy of the individual small fibers.

8. It is proposed that the concept of cardiac "atrophy" is a chimera and that it be replaced by the term "hypocardia."

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THE EFFECTS OF ANIMAL SERUMS ON LYMPHOSARCOMA 6C3HED IN C3H MICE *

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During the course of studies on resistance and immunity in transplantable cancer, Kidd,[1] in 1953, discovered that normal guinea pig serum, when administered intraperitoneally, brought about regression of lymphosarcoma 6C3HED in C3H mice and lymphoma II in A strain mice and delayed the appearance of Murphy-Sturm lymphosarcoma in Wistar rats. The serum had no effect on sarcoma 180 in C3H mice or on 2 types of mammary carcinoma also carried in C3H mice. Extending his studies, he noted that horse and rabbit serums possessed no tumor inhibitory properties against lymphosarcoma 6C3HED, lymphoma II, and Murphy-Sturm lymphosarcoma, but that human serum temporarily, albeit slightly, inhibited growth of similar lymphosarcoma cells *in vivo*.

In attempting to explain the natural resistance of laboratory animals to heterologously transplanted tissues in general, and to human tumors in particular, we[2] theorized that the properdin system might be responsible for this innate protective mechanism and that the tumor inhibitory principle (TIP) in guinea pig serum might actually be properdin. Basing our procedure on the findings of Pillemer and Ross,[3] we[4] successfully conditioned weanling Wistar rats to a transplantable human carcinoma of the colon (HR132) with zymosan, thus obtaining good tumor "takes" in a high proportion of animals implanted with the cancer. Subsequent experiments,[5] however, indicated that the TIP in guinea pig serum was probably not properdin. In further attempts to identify this principle, we were led to conclude that it was likewise neither complement nor polysaccharide.[6]

It is obviously of interest to determine whether the tumor inhibitory principle is peculiar to guinea pig serum or whether serum from other animals may also contain the substances. This would be especially true in connection with larger animals, such as the cow or sheep, which could serve as far more adequate and less expensive sources of serum for investigative purposes than the guinea pig. For this reason, and because the work of Kidd[1] concerning serum from animals other than the guinea pig has, to our knowledge, neither been substantiated nor extended, the following studies were undertaken.

* Received for publication, November 18, 1957.

Materials and Methods

The tumor-host combination in each of the experiments consisted of the Gardner lymphosarcoma 6C3HED and female C3H mice. The animals weighed in the neighborhood of 20 gm. each and were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The donor tumor was 9 days old and was implanted into the subcutaneous tissues of the right flank by the usual trocar technique. Treatment with one or another substance was begun 4 days after implantation when the tumors were approximately 8 mm. in diameter.

All guinea pig serum, as well as other serums and sheep plasma marked (C) in Table I, were purchased in the lyophilized state from Cappel Laboratories, West Chester, Pennsylvania. All rat serum, also in the lyophilized state, was purchased from Carworth Farms, New City, New York. Serums marked (S) were obtained from the Stine Laboratory, E. I. duPont deNemours & Company, Newark, Delaware, and, except for the sheep serum in Experiment 4 which was lyophilized, they were all received in the fresh state. Regardless of the source, lyophilized serums were restored with sterile distilled water either to normal volume (N.) or to such volume (conc.) that 0.2 ml. was equivalent to 0.67 ml. of normal serum. The human serum and plasma were both obtained from the same individual with group O, Rh positive blood.

In each experiment a group of tumor bearing mice was set aside as nontreated controls while another group was treated with guinea pig serum (g.p.s.). All treatment was administered intravenously by way of tail veins and was given either daily or 3 times a week (3 x's wk.). In two experiments (2 and 3) serum was saved from treated mice for properdin determinations. These were kindly performed for us by Dr. Louis Pillemer. All surviving animals were sacrificed 14 days after implantation of tumor. A necropsy was performed on each, and the tumor or tumor site, liver, and a kidney were examined microscopically. As in experiments reported elsewhere,[5,6] however, the gross rather than microscopic disappearance of the tumor was taken as evidence of complete regression.

Results

The type and frequency of treatment, the number of animals used, and the results obtained are outlined in Table I.

Experiment 1. The same dose, concentration, and frequency of injection of rat serum as of guinea pig serum was administered to 15 mice. It will be noted that nontreated animals showed progressive growth of tumor, that all animals treated with g.p.s. disclosed complete

TABLE I

Experimental Data

Treatment, Number and Size of Tumors, and Weights of Animals

Experiment	Treatment*	No. mice used	No. mice surviving	No. with tumors	Average diameter tumor	Average weight of animal	
						Initial	Terminal
1	None	15	15	15	26 mm.	20 gm.	33 gm.
	0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5†	15	15	0	0	24	27
	0.2 ml. conc. rat s. 3 ×'s/wk. × 5	15	15	15	25	24	35
2	1.0 ml. N. saline daily × 6	2	2	2	28	24	34
	1.0 ml. N. g.p.s. daily × 6	4	4	0	0	24	24
	1.0 ml. N. rat s. daily × 6	5	5	5	24	23	30
	1.0 ml. N. human s. daily × 6	5	5	5	24	24	31
	1.0 ml. N. sheep s. (S) daily × 6	5	5	3	14	25	25
	1.0 ml. N. chicken s. (S) daily × 6	5	5	5	23	23	29
	1.0 ml. N. dog s. (S) daily × 5	5	5	5	21	26	30
	0.4 ml. N. bovine s. (S) daily × 9	5	5	5	25	26	32
3	None	15	12	12	26	20	26
	0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5	15	15	0	0	22	22
	0.45 ml. conc. sheep s. (C) 3 ×'s/wk. × 5	15	14	14	23	22	25
	0.3 ml. conc. sheep plasma (C) 3 ×'s/wk. × 5	15	12	12	26	22	30
	0.9 ml. conc. horse s. (C) 3 ×'s/wk. × 5	15	13	13	24	22	25
	0.13 ml. conc. bovine s. (C) 3 ×'s/wk. × 5	15	15	15	26	22	29
	0.3 ml. conc. calf s. (C) 3 ×'s/wk. × 5	15	14	14	25	21	29
	0.08 ml. conc. rabbit s. (C) 3 ×'s/wk. × 5	15	15	15	25	21	31
	1.0 ml. conc. rat s. 3 ×'s/wk. × 5	15	13	13	25	23	30
	0.67 ml. N. human s. 3 ×'s/wk. × 5	15	15	13	19	16	21
	1.0 ml. N. human plasma 3 ×'s/wk. × 5	15	15	15	24	21	28
4	None	10	10	10	13	23	22
	0.2 ml. conc. g.p.s. 3 ×'s/wk. × 2	10	10	7	4	17	18
	0.3 ml. conc. sheep s. (C) daily × 3	10	9	9	10	19	18
	0.3 ml. conc. sheep s. (S) daily × 3	10	10	10	11	17	17
	1.0 ml. N. sheep s. (S^1) daily × 3	10	10	10	12	19	18
	1.0 ml. N. sheep s. (S^2) daily × 3	10	10	10	13	19	18
	0.67 ml. N. calf s. (S) daily × 3	10	10	10	10	17	16
	0.3 ml. conc. calf s. (C) daily × 3	10	8	8	12	17	18

* See text for abbreviations and symbols.

† Indicating injections 3 times a week for 5 injections.

regression of tumor. Mice treated with rat serum exhibited no retardation of growth of the tumor.

Experiment 2. Although fewer animals were used, a greater variety of serums was tested. The dosages of guinea pig and rat serums were increased; the amounts of other serums were used at maximum tolerated dosages, and injections were given daily over 6 consecutive days. Since 1.0 ml. is quite a large amount of fluid to introduce intravenously into a mouse, it should be noted that 2 control animals received 1.0 ml. of physiologic saline without deleterious effect. Actually, in other experiments not recorded, as much as 5 ml. of physiologic saline was administered intravenously as a single injection with no apparent harmful effect on the animals. As would be expected, increasing the dosage as well as the frequency of administration of g.p.s. yielded complete regression of neoplasm in 4 of 4 animals so treated. As noted in Table I, no tumor inhibitory effect was induced by rat, human, chicken, or dog serum given in doses of 1.0 ml. or by bovine serum given in doses of 0.4 ml. Sheep serum, however, in doses of 1.0 ml. resulted in complete regression of tumor in 2 of 5 animals, and the average diameters of the 3 tumors that grew were 14 mm. as compared with 28 mm. in the corresponding saline treated controls. Microscopic examination of the liver from all animals in this experiment and of the kidneys from animals treated with guinea pig, rat, human, chicken, dog, and bovine serums showed no noteworthy alterations. The kidneys of mice treated with sheep serum revealed toxic nephrosis, as evidenced by extensive tubular degeneration, necrosis, and the appearance of numerous pigment casts in the tubule lumens.

Properdin titers (units per ml.) as determined in the serums used in this experiment were guinea pig serums (normal), 3 units; rat serum, 30 units; human serum, 6 units; sheep serum, 18 units; bovine serum, 18 units; chicken serum, less than 1 unit; and dog serum, 3 units. The titers in serums from animals used in the experiment were as follows: (1) normal mice, nontumor bearing, 9 units; (2) 4 nontreated mice showing progressive enlargement of tumor, 6, 6, 4, and 1 units; (3) mice receiving guinea pig serum and exhibiting regression of tumor, 3 units; (4) mice receiving rat serum and with continued growth of the implanted tumor, 6 units; (5) mice treated with human serum without effect on tumor growth, less than 1 unit; (6) mice treated with sheep serum and exhibiting regression of tumor, 6 units; (7) mice receiving chicken serum with tumor growth continuing, 3 units; (8) mice treated with dog serum with unaffected tumor, 6 units; and (9) mice receiving bovine serum with continued tumor growth, 3 units. In other publications we[5,6] have shown that the properdin level of serums from C_3H

mice bearing lymphosarcoma 6C3HED varied inversely with the growth of tumor regardless of the type of treatment. The properdin titers of serums from animals in this experiment substantiated our previous findings with 2 exceptions. One of these was the observed level of 3 units in animals treated with g.p.s. and showing regression of tumors. One would have expected the reading to be normal (9 units) or above. For this, we have no explanation. As a matter of fact, in a parallel experiment, properdin values in serums obtained serially from animals treated with concentrated g.p.s. and showing regression of tumors were 24, 18, 9, and 9 units. The other exception encountered occurred in the serum of animals treated with sheep serum and showing regression of tumor. The observed properdin level of 6 units was expected to have been normal (9 units) or above. One possible explanation for the low reading might have been the toxic nephrosis which appeared in these mice.

Experiment 3. In an attempt to get a more uniform picture, a greater number of animals was employed in each group and, with the exception of human serum and plasma, lyophilized rather than unprocessed serum and plasma were utilized. Once again, a maximum tolerated dose for each material on hand was used in the experiment. As noted in Table I, all animals among the nontreated controls that survived 11 days or longer (the others were sacrificed for properdin determinations) showed progressive growth of tumor. Fifteen mice treated with concentrated g.p.s. revealed regression in each instance. Animals treated with sheep serum, sheep plasma, horse serum, bovine serum, calf serum, rabbit serum, rat serum, and human plasma revealed steady and progressive growth of tumor comparable to that in nontreated controls. Among the 15 mice treated with human serum there was regression of tumor in 2, and the average diameter of the remaining lesions that did grow was 19 mm. as compared with 26 mm. in nontreated controls. Microscopic studies again revealed toxic nephrosis in mice treated with sheep serum. The kidneys and livers from other animals were normal.

Properdin titers (units per ml.) in serums used in this experiment were as follows: guinea pig serum (concentrated), 12 units; sheep serum, 72; horse serum, 25; bovine serum, 24; calf serum, 36; rabbit serum, 48; rat serum, 60; and human serum, 6 units. The titers in serums from animals used in the experiment were as follows: (1) normal mice, nontumor bearing, 12 units; (2) nontreated mice with large tumors, 3 units; (3) mice receiving guinea pig serum and showing regression of tumor, 18 units; (4) mice treated with sheep serum, 3 units; (5) mice treated with sheep plasma, 3 units; (6) mice re-

ceiving horse serum, 3 units; (7) mice treated with bovine serum, 3 units; (8) mice receiving calf serum, 3 units; (9) mice treated with rabbit serum, 3 units; (10) mice treated with rat serum, 3 units; (11) mice treated with human serum, 3 units; (12) mice receiving human serum with regression of tumors, 9 units; and (13) mice receiving human plasma, 3 units. With the exception of those in groups 1, 3, and 12, all of the above groups of mice showed large tumors at the conclusion of the respective experiments. Once again, as in other studies,[5,6] these results indicate that the properdin level of serum in C_3H mice bearing lymphosarcoma $6C_3HED$ varies inversely with growth of tumor.

Experiment 4. Although conditions in Experiments 2 and 3 were not exactly parallel, the fact that sheep serum at first did and then did not show tumor inhibitory properties was disturbing. While Experiment 4 was primarily conducted to retest sheep serum, calf serum was also included because it was available. In this experiment, concentrated sheep serum (C) and concentrated calf serum (C) were obtained from the same respective lots used in Experiment 3; concentrated sheep serum (S) was lyophilized from the same lot used in the next group and is labeled "N. sheep s. (S^1)" in Table I, while the second sample of "N. sheep s. (S^2)" was obtained from the same lot used in Experiment 2 and kept frozen on dry ice from the time it was collected. As a matter of fact, all sheep serum in this group marked (S) was from the same sheep but procured on 2 separate occasions. While the tabulations in the first 3 experiments in Table I are based upon animal survival of 11 days or longer, the notations in Experiment 4 depict circumstances as they existed 6 days after implantation of tumor, for many of the animals in the last 4 groups died after the third injection. By this time, however, the trend was considered well enough established to indicate that none of the samples of sheep or calf serum had any tumor inhibitory properties. It might be stated in passing that the first 4 groups in Experiment 4 were brought to 11+ days of completion and disclosed good growth of tumor in each of the nontreated controls, complete regression of tumor in all of the mice treated with concentrated g.p.s., and no tumor inhibition in animals treated with lyophilized (C) or (S) serum.

Discussion

In assembling the facts gleaned from these experiments, it may be stated that the Gardner lymphosarcoma in C_3H mice continues to be vulnerable to treatment with guinea pig serum. Among 44 mice thus treated, all showed complete regression of tumor. It may also be stated

that, in the amounts used, none of the following serums exhibited any tumor inhibitory properties: rat, chicken, dog, bovine, horse, calf, and rabbit. Negative results were obtained also with sheep plasma and human plasma. While in one experiment sheep serum brought about 2 regressions and 3 retardations of growth in 5 animals treated and in another, human serum produced 2 regressions and 13 retardations of growth in 15 animals treated, these results are not considered significant. They could possibly indicate, however, that a tumor inhibitory principle (TIP) may be present in serum from animals other than the guinea pig although in much smaller amounts. If and when the TIP from guinea pig serum is isolated and concentrated, the application of a similar method to serums from other animals may prove fruitful.

Properdin determinations in serums from animals treated with serum and plasma from animals listed, as in other reports,[5,6] indicated in general that properdin titers were low when the tumors were large, and normal or elevated when they had regressed. From this, it would appear that the animal's own properdin system was involved either in controlling the growth of the transplanted tumor or at least in reflecting its activity. One final item which deserves mention is that the TIP in guinea pig serum is probably not properdin since all serums in the experiments recorded, except chicken serum, possessed properdin titers equal to or many times higher than that of guinea pig serum and yet none of them showed the tumor inhibitory activity of guinea pig serum.

Summary

Serums from several types of animals were tested for tumor inhibitory activity against the Gardner lymphosarcoma 6C3HED carried by C3H mice. Complete inhibition of growth was obtained with guinea pig serum; slight and inconstant inhibition was demonstrated with sheep and human serums, and no inhibition was noted with rat, chicken, dog, bovine, horse, calf and rabbit serums, or with sheep or human plasma.

Properdin titers in serums from mice treated with serums from the animals listed varied inversely with the growth of the tumor. Because of this, it was concluded that the animal's own properdin system was involved either in controlling the growth of the transplanted tumor or in reflecting its activity.

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PRODUCTION OF SALIVARY GLAND VIRUS INCLUSIONS IN CULTURES OF MOUSE SALIVARY GLAND *

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The salivary gland virus (SGV) of many rodents and monkeys is a transmissible agent and apparently is species specific.[1] The inclusion bodies it produces resemble those produced by the virus of the human salivary gland disease of infants and adults.[2-4] However, the transmission of the human SGV to animals has not been achieved.[3-5] In the salivary glands of man and guinea pigs, virus inclusion bodies occur mostly in the ductal epithelium,[2] while in the mouse they are found in the acinar epithelium.[6]

The course of infection in mice is dependent upon the route of introduction of the virus. When it is given intraperitoneally, most of the animals die within 4 to 7 days. On the fifth day after a lethal dose, the animal undergoes marked and rapid changes in appearance, becoming hunchbacked with obvious abdominal discomfort, and exhibiting ruffled fur. Within 12 hours after the onset of these symptoms, the animal usually dies. This threshold of manifest toxic effects is concomitant with the formation of SGV inclusion bodies. Inclusion bodies appear consistently in the liver, spleen, and adrenals. They are not found in the salivary gland, although we have recovered the virus by subsequent transfers of salivary gland suspensions. In animals which survive and are permitted to live for two weeks, the salivary glands usually contain inclusion bodies. If the SGV suspension is introduced subcutaneously, the viscera do not show inclusion bodies, and the animals do not die. SGV inclusion bodies appear in the salivary glands as early as the seventh day and disappear between the fourth and eighth weeks.[7]

The development of inclusion bodies in the salivary glands of the living animal can be reconstructed only from the sequence of histologic events observed in mice sacrificed at different time intervals after SGV administration. The cell affected by SGV increases in size and appears round or oval; its cytoplasm is often granulated, while the nucleus becomes hyperchromatic. Later the nucleus contains a somewhat centrally located inclusion body, which is separated from the nuclear membrane by a clear zone. In the early stages of inclusion body for-

* This investigation was supported in part by Research Grant D-785 from the Division of Research Grants, National Institutes of Health, U.S. Public Health Service.

Received for publication, November 21, 1957.

mation, this "halo" is narrow, and the ground network of the nucleus is retained. Later, however, the chromatin collects at the periphery of the nucleus, thus widening the "halo." The cytoplasm of the cells containing inclusion bodies appears foamy. In the final stages, the inclusion body has increased in size, and the nuclear membrane is ruptured. Occasionally one gets the impression that the inclusion bodies have developed in the nuclei of a binucleated cell ("twin inclusion bodies").

In some cells, cytoplasmic inclusions occur.[8,9] These are basophilic and appear different histochemically from the intranuclear virus inclusions.

While it has been demonstrated that in the living animal the epithelial cell of the salivary gland is the residual host to the SGV, the pathogenicity of the virus to salivary glands in tissue culture has not been established. Andrewes[10] reported that in tissue cultures of guinea pig and mouse gland tissue, the SGV inclusions were constantly observed in histiocytes after exposure to this virus. Smith[1] reported that she had propagated the mouse salivary gland virus in growing embryo tissue with the production of SGV inclusion bodies.

The purpose of the present study is to describe the process of formation of SGV inclusion bodies in the cells of cultures of mouse salivary gland.[11]

Material and Methods

Tissue Explants. Under ether anesthesia, the salivary glands were removed aseptically from 3- to 5-day-old mice, immediately placed in Petri dishes containing Tyrode's solution, and cut into approximately 1 mm.[3] pieces. The mice used in these experiments were of the C (BalbC) and CFW strains. These strains have never been found to be natural carriers of the SGV.[6]

Salivary Gland Virus (SGV) Suspensions. The mouse SGV used in these experiments had originally been supplied to us by Dr. Margaret Smith of Washington University, School of Medicine (Smith strain). We have maintained a stock of this virus in mice by serial subcutaneous injections (every 2 to 3 weeks) of suspensions of virus-infected salivary gland. (a) Viable virus suspension: The SGV suspensions were prepared by grinding several salivary glands of the SGV stock donors in 5 to 10 per cent fine pumice and Tyrode's solution (one gland per ml. of solution). These suspensions were centrifuged at 2,000 r.p.m. for 15 minutes and the supernates removed, frozen in the refrigerator freezer compartment and thawed in air at room temperature. All viable SGV suspensions were positive for the SGV on bioassay. (b) Heat treated virus suspensions: Aliquots of viable SGV

suspensions were placed in a water bath for 30 minutes at 56° to 60° C. The heating of SGV suspensions inactivated the virus, as ascertained by bioassay. These "nonviable virus" suspensions were used for control cultures.

Nutrient Medium.[12] (a) Chick embryo extract: Eight-to 10-day-old check embryos were macerated in Petri dishes containing Tyrode's solution (one embryo per 2 ml. of solution), forced through a syringe into centrifuge tubes, and centrifuged at 2,000 r.p.m. for 20 minutes. The supernates were removed, placed in the freezer for several hours and then slowly thawed at room temperature. This freezing and thawing was repeated before using. (b) Mouse embryo extract: Mouse embryos less than 20 days of age were removed from the uteri of either C or CFW mice and placed in Petri dishes with Tyrode's solution (one embryo per 10 ml. of solution). The embryos were homogenized in a Waring blender, and the resulting homogenate was centrifuged at 2,000 r.p.m. for about 20 minutes. The supernates were removed and twice frozen and thawed. A combination of chick and mouse embryo extracts in a 1:1 ratio was used as the nutrient medium.

Fowl Plasma.[12] Blood was withdrawn from a cock (starved for 24 hours), by cardiac puncture, into a syringe containing heparin in a final concentration of approximately 0.3 mg. of heparin per ml. of whole blood. The blood was centrifuged, and the plasma was removed, placed in small vials, and kept in the refrigerator (not frozen).

Tissue Culture Methods. (a) Maximow's double cover slip method: One drop of fowl plasma and one drop of nutrient medium were dropped in the center of a cover slip and 2 to 3 tissue explants placed into this mixture before clotting. A large depression slide was used to cover the cover slip assembly. It was sealed with paraffin and the entire preparation placed in the inverted position in a slide rack and kept at 37° C. (b) Leighton's sponge matrix roller method:[13] Cellulose sponge served as a matrix which passively held the tissue cells in place, while in the double cover slip method the only matrix was the fibrin of the fowl plasma and eventually even this fibrin matrix liquefied. Commercial cellulose sponge (DuPont) was cut with a razor blade into approximately 1 by 5 by 10 mm. pieces. The sponge pieces were washed as described by Leighton,[13] and the sponge strips were stored in distilled water in the refrigerator. Before use, a sponge strip was placed into a 150 by 16 mm. glass culture tube with a constricted neck (10 mm. in diameter), and autoclaved for 15 minutes. Several explants of the salivary gland tissue were carefully placed on the surface of the sponge previously moistened with fowl plasma. One drop of the nutrient medium was added to the sponge surface to accelerate

the clotting process. The sponge strip with the attached explants was bathed in 0.5 ml. of the nutrient medium. Then the sealed culture tubes were placed in a roller tube drum (12 r.p.h.) and kept at 37° C.

Virus Inoculations. Only SGV suspensions which were no older than 24 hours were used in these experiments. The virus was introduced into growing (2- to 3-day-old) tissue cultures with a wire loop, 3 mm. in diameter. One set of cultures received "viable" SGV suspension, while another set, serving as controls, received "nonviable," heated SGV suspension.

Bioassays. Normal young C or CFW mice were used as test animals and received 1 ml. of the test fluid intraperitoneally. A bioassay was regarded as positive if within 2 weeks SGV inclusion bodies were found in either the viscera or salivary glands. All nutrient media, SGV suspensions, and Tyrode washings of both control and experimental tissue cultures were bioassayed. Washings of the cultures were obtained by immersing the tissue explants in 5 ml. of Tyrode solution. On several occasions tissue explants suspended in Tyrode solution were also assayed.

Histologic Procedures. The control and virus cultures of both methods were washed with Tyrode and then fixed in either 10 per cent formalin or Zenker's fluid with acetic acid. The cultures on cover slips were either stained *in toto,* or the explants were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The explants attached to the sponge matrix were embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Experimental Schedule. Three separate experiments were performed with fresh nutrient media, tissue explants and SGV suspensions. In each experiment, 20 to 30 cultures prepared by each of the two techniques were employed. The SGV (viable and nonviable) was introduced into the tissue cultures after 2 to 3 days of incubation. Two to 3 cultures were used for cytologic examinations every day for 10 days.

Results

Control Cultures. Explants grown by Maximow's double cover slip culture technique showed very good proliferation and migration of fibroblasts and epithelial cells into the periphery. Satisfactory results were also obtained with Leighton's sponge matrix method, which has the advantage of permitting the tissue explants to continue as organized tissues and to retain the basic morphologic patterns observed *in vivo.* Heated, "nonviable," SGV suspension added to these cultures did not alter the tissue explants detectably. All controls were negative by bioassay for the SGV.

Experimental Cultures. In the cultures prepared by the double cover slip method, the addition of unheated ("viable") SGV suspensions resulted in a profound alteration of the growing explants. No changes were observed during the first 4 days following the introduction of the virus, the cells proliferating and migrating as before inoculation. About the fifth day, the clotted matrix liquefied rapidly, the peripheral cells lost intercellular cohesiveness, the cells became rounded in shape, and the cytoplasm appeared granular in the unstained cultures (Fig. 1). In the stained cultures, many intranuclear inclusion bodies were found in these migrating cells.

At the time when the peripheral cells of the explant first showed inclusion bodies, the centrally located cells were free of these, but contained eosinophilic, cytoplasmic condensations of various sizes (Fig. 2). At a later stage the cells in the central area of the explant also developed intranuclear inclusion bodies. This stage was rapidly followed by complete disintegration of the explant. The eosinophilic, cytoplasmic condensations appeared isolated from the well preserved nuclear membrane. Similar condensations were occasionally observed in cells of the control cultures. The peripheral cells of the infected cultures did not show cytoplasmic condensations at any time.

The cultures prepared by the sponge matrix technique, when exposed to "viable" SGV suspension, also revealed a marked disintegration of the explant in stained sections (Fig. 3) and large numbers of intranuclear SGV inclusion bodies appearing as early as the fifth day after inoculation. As with the Maximow technique, the inclusion bodies were found in the peripheral cells of the growing explants and gradually decreased in number toward their centers. The eosinophilic, cytoplasmic condensations were also observed in cultures prepared by this method, but their numbers were not as impressive as in the cover slip preparations.

With both the Maximow (Fig. 4) and Leighton (Fig. 5) methods, binucleated cells with a virus inclusion body in each nucleus ("twin" intranuclear inclusion bodies) were seen. Their number was particularly great in the more peripheral migrating cells.

In the cover slip preparations it was occasionally noted that one or two tissue explants embedded in the same plasma clot, although exposed to SGV, did not display tissue response, as indicated by either the loss of intercellular cohesiveness or by inclusion body formation. Failure of infection was not observed in cultures prepared by Leighton's method. This may be explained by the fact that in the sponge matrix technique, the nutrient fluid is in constant motion and the virus particles are presumably evenly distributed to all explants, whereas in

the cover slip method the fluid is entrapped in the fibrin matrix of the plasma clot, and therefore the nutrient is almost static.

In contrast to the ease of infecting the tissue cultures 3 days after explantation, it was difficult to infect the cultures on the first day of growth.

Discussion

Salivary gland tissue explants may be infected readily by suspensions of salivary gland virus and exhibit the production of typical inclusion bodies. The pathogenic potency of the virus for the cells of the explanted glands is evident from the repeated success in infecting the cultures and from the large number of proliferating cells which showed typical inclusion bodies. That inclusion bodies develop first in the peripheral cells of the explant is perhaps due to the fact that these cells are closer to the surface upon which the inoculum is being placed.

The infection exhibits a threshold of "toxic" effect, which is found to be concomitant with the formation of SGV inclusion bodies both *in vivo* and in tissue culture. *In vivo*, this threshold is usually passed by the fifth day after a lethal dose, when systemic signs become evident. In tissue culture, the "toxic" effect of the virus is manifested by the disintegration of the explants. This also occurs at nearly the same time on the fifth day after inoculation.

Following subcutaneous inoculation of mice with SGV, occasionally two inclusion bodies appear to be present in binucleated salivary acinar cells. However, it is difficult to decide whether compression of two adjacent cells in a limited space may not simulate twin nuclei with inclusions, or whether binucleated cells are actually present. In the infected tissue cultures observed in the present study, binucleated cells, with each nucleus containing an inclusion body, were identified clearly and frequently (Fig. 5). It is possible that twin nuclei with inclusions arise when the virus invades dividing cells and interrupts the cleavage of the cytoplasm which follows nuclear division; thus inclusion bodies appear in each of the two nuclei. Strangeways[14] has explained the formation of binucleated and multinucleated cells in tissue culture as an alteration in mitotic division due to unfavorable conditions. Smith[1] observed an occasional cell containing 2 or 3 intranuclear inclusion bodies in 1 cell. Ivanovics and Hyde[15] reported that in regions containing great numbers of inclusions, mitosis was not observed. On the other hand, amitotic division occurred frequently even in cells bearing inclusions, and many cells with more than one nucleus resulted. The fact that the salivary gland tissue is proliferating rapidly in culture would account for the fact that twin inclusion bodies were more numerous in tissue culture than *in vivo*. "Twins" are more

numerous in the marginal cells of the explants, where cell division predominates.

One may only speculate as to the significance of the eosinophilic condensations in the cytoplasm. Pearson[8] and Rosenbusch and Lucas[9] observed cytoplasmic inclusions in infections induced *in vivo*. These were basophilic and developed later than the intranuclear inclusion bodies. The authors thought that these inclusions were the result of some type of injury to the cytoplasm, attributable to the virus infection. The cytoplasmic inclusions observed in the tissue cultures are unlike those observed *in vivo;* they are acidophilic and occur before the development of the intranuclear bodies. However, they probably represent a similar type of direct injury to cytoplasm by the virus or by alterations in metabolism caused by the infection.

Summary

Cultures of mouse salivary gland tissue were grown, using both Maximow's double cover slip and Leighton's sponge matrix methods. The cultures were exposed to a salivary gland virus (SGV) suspension 3 days after explantation. Infection of the experimental cultures was invariably successful in cultures grown by Leighton's method and successful in the majority of explants grown according to Maximow's method. Numerous typical intranuclear SGV inclusion bodies appeared within a few days, first in the migrating and proliferating peripheral cells and later in the more centrally located elements. This stage was followed by complete disintegration of the cultures. Infected cultures revealed numerous twin inclusion bodies in binucleated cells. Tissue cultures exposed to heated SGV suspensions as controls were unaffected, exhibiting no inclusion body formation in the cultures or on bioassay.

An incubation period of 5 days intervened between inoculation of the cultures and the appearance of manifest "toxic" effects. This latent period was of the same duration as that following the intraperitoneal administration of a lethal dose of SGV suspension to mice.

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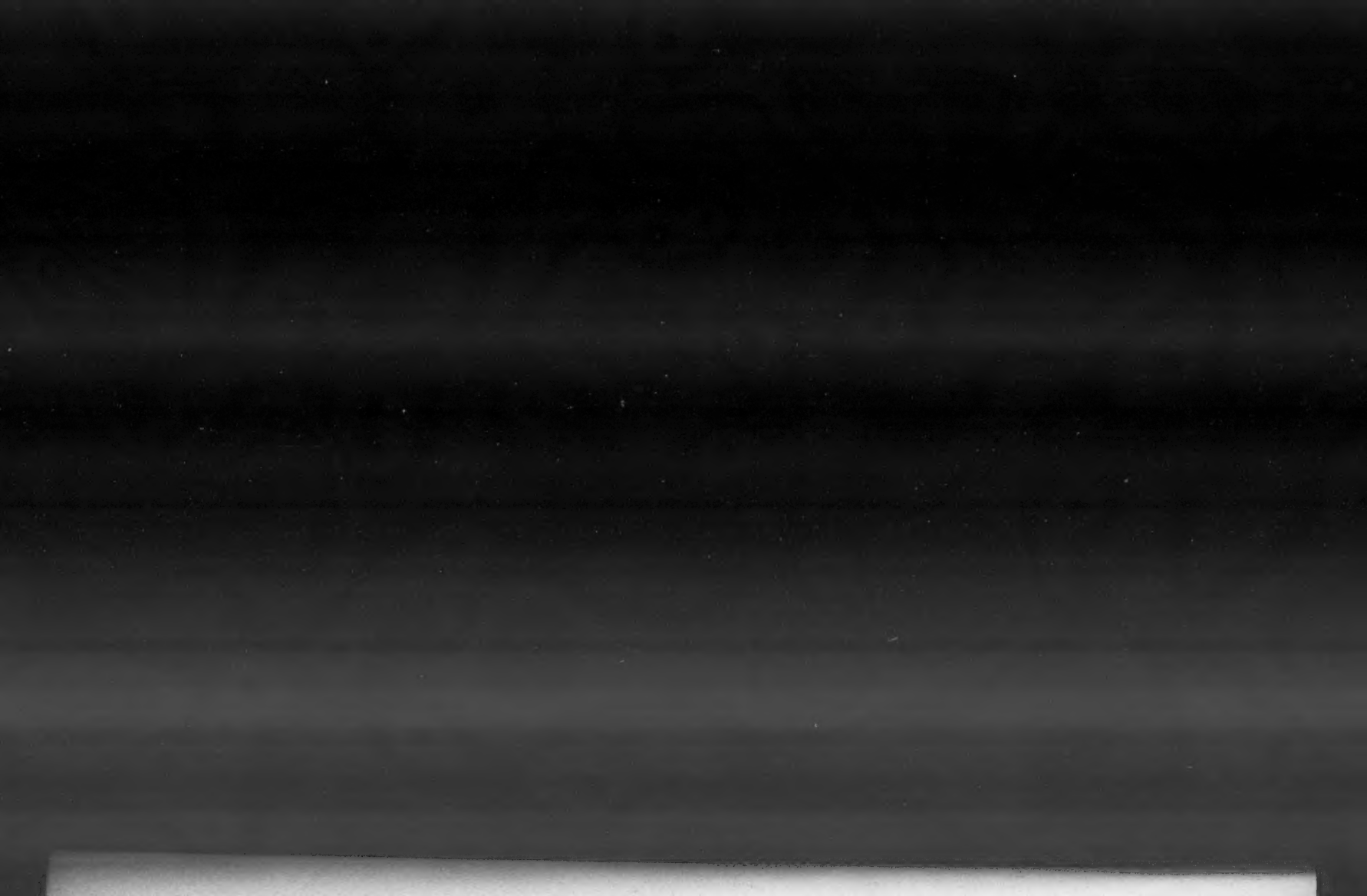
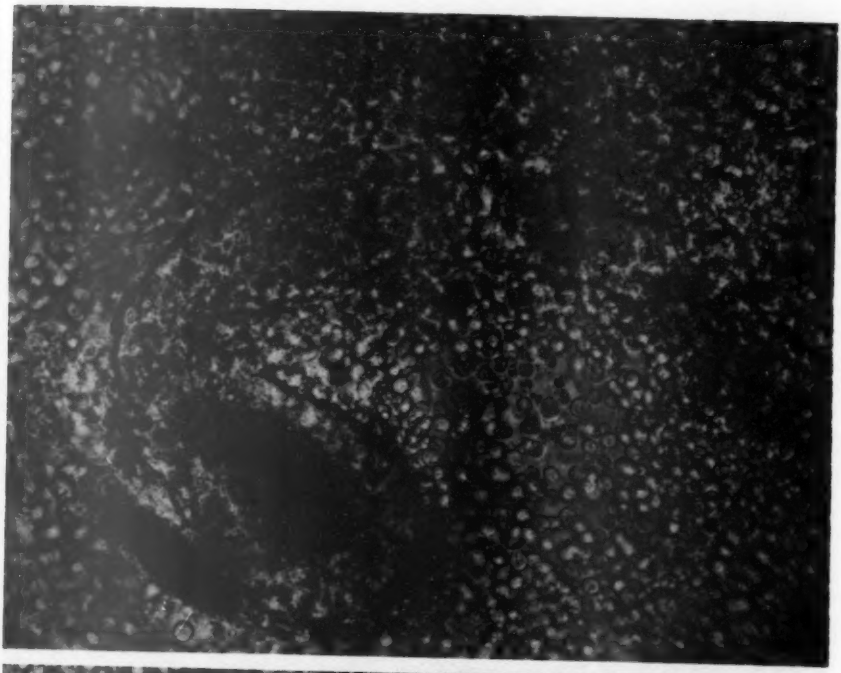
LEGENDS FOR FIGURES

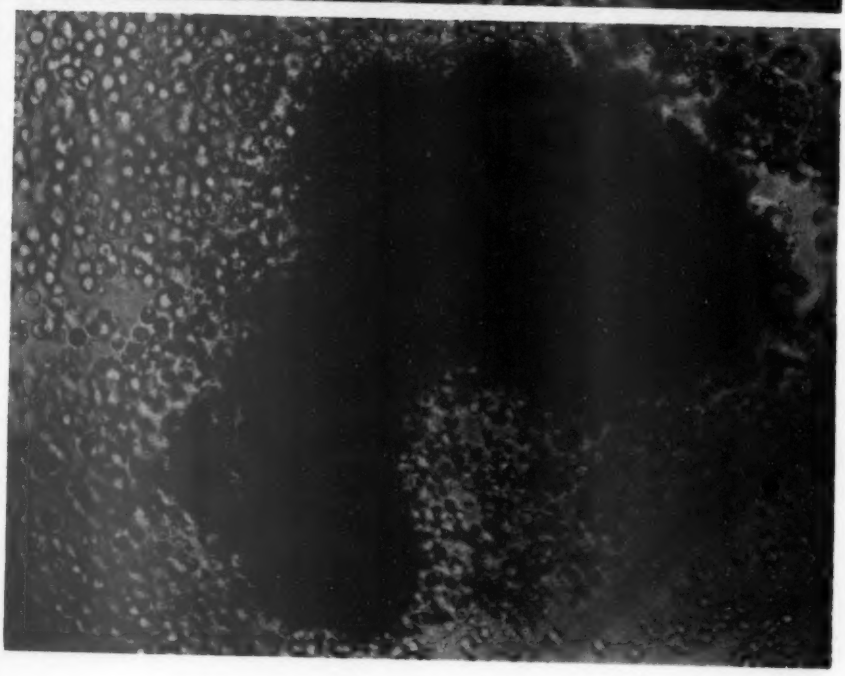
FIG. 1a. A 7-day salivary gland explant (Maximow culture) exposed to SGV. Note proliferating and migrating cells. The end portion of a duct is occluded, and the lumen is dilated with fluid. The duct and its branches are disintegrating. Note the loss of cellular cohesiveness and rounding of the cells. Unstained. × 600.

FIG. 1b. Three days later. Note extensive disintegration and necrosis of the explant. Unstained. × 600.





1 A



1 B

FIG. 2. Several peripheral migrating cells of a SGV-exposed 7-day salivary gland explant (Maximow culture). The center cell contains a typical "cytoplasmic condensation" without intranuclear inclusion. Hematoxylin and eosin stain. × 1,400.

FIG. 3. Salivary gland explant (Leighton culture) 6 days after exposure to the SGV. Note the loss of cellular cohesiveness and areas of necrosis. Hematoxylin and eosin stain. × 400.

2

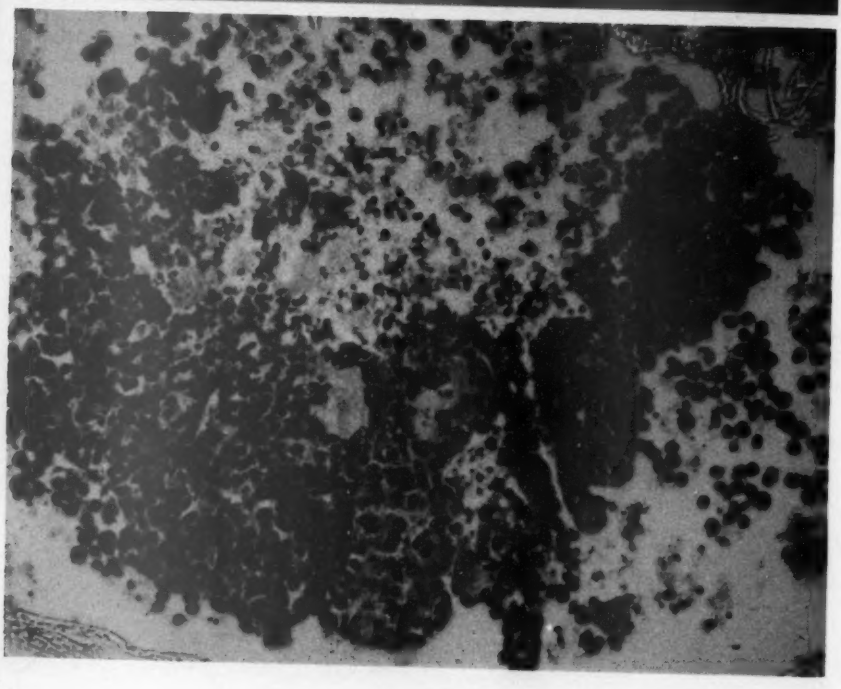


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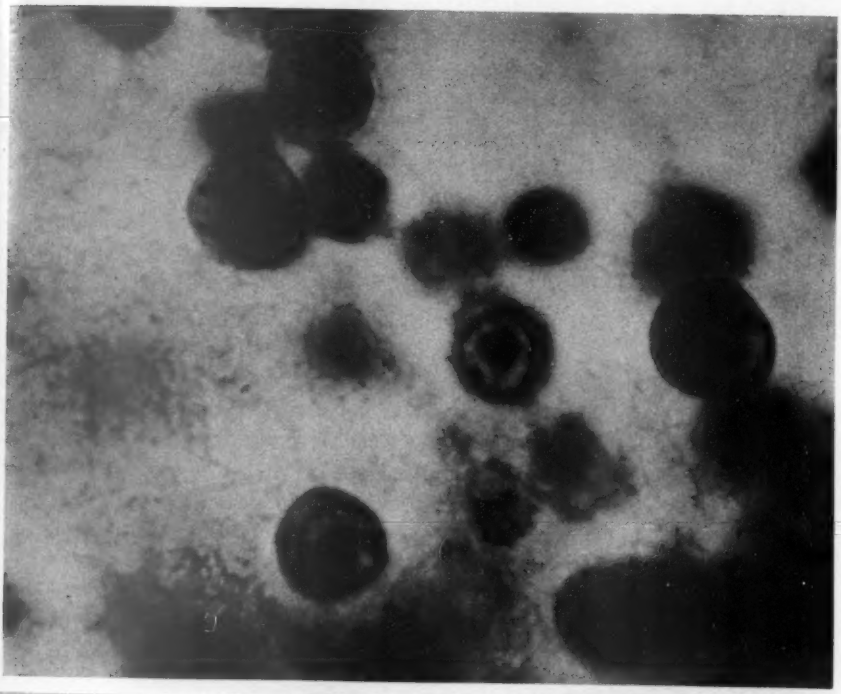
FIG. 4. A peripheral area of a SGV infected salivary gland explant, showing intranuclear inclusions. Note the atypical mitoses. (Maximow method.) Hematoxylin and eosin stain. × 1,200.

FIG. 5. Marginal area of a SGV-exposed salivary gland explant (Leighton culture) 9 days after explantation. Note numerous "twin inclusions" in daughter nuclei. Hematoxylin and eosin stain. × 2,000.



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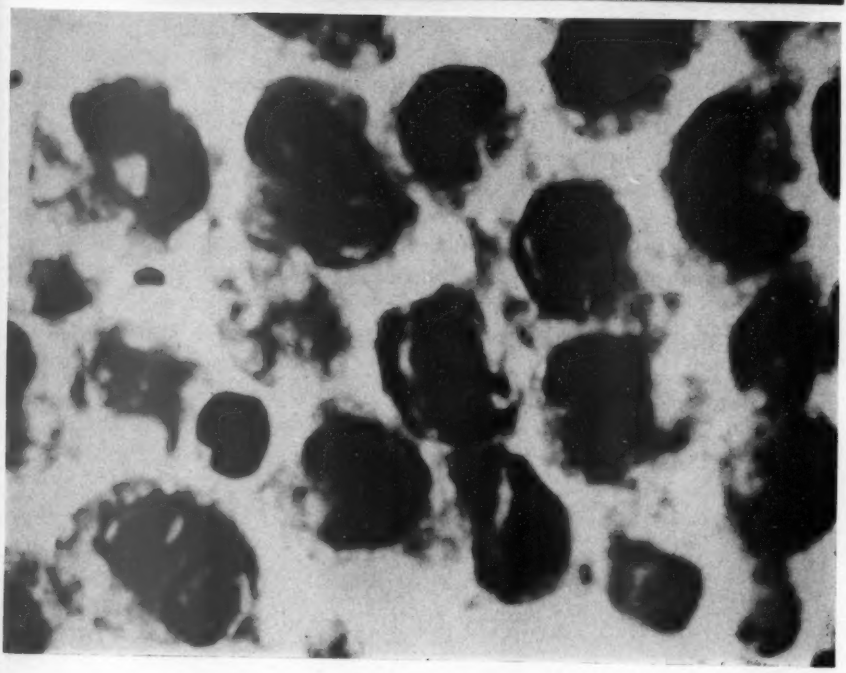

5

CELLULAR LESIONS IN MONKEY KIDNEY EPITHELIUM INDUCED BY ADENOVIRUSES AND VIRUSES OF SIMIAN ORIGIN *

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The intracellular changes produced by adenoviruses have been described in detail by Barski,[1] Lépine, Chany and Robbe-Fossat,[2] Boyer, Leuchtenberger and Ginsberg,[3] Bloch, Morgan, Godman, Howe and Rose.[4] In most of these studies HeLa cells were used. It has been established that the significant primary changes, as observed with the light microscope, take place in the nucleus, and that such changes are characteristic of this particular group of viruses.

The use of monkey kidney cells in culture frequently is complicated by the appearance of so-called "simian viruses."[5] Some of these agents produce cytologic changes that are similar to those produced by adenoviruses when propagated in cell culture. This work was undertaken to study further the cellular alterations induced by adenovirus in monkey kidney epithelium and to compare these with those induced by some simian viruses.

Boyer and co-workers[3] have described virus-induced lesions in HeLa cells, and Beale, Doane and Ormsby,[6] in human amnion cells. Many of these basic alterations also were noted in this work. It seems necessary, therefore, only to discuss differences encountered in our observations and extensions of reported data.

Materials and Methods

Cells. Sheets of monkey kidney epithelium were grown on 22 by 24 mm. cover slips that were placed in 6-ounce, wide mouth, French Square bottles. One cover slip was placed in each bottle. Trypsinized cells (0.5 ml. of a 1:10 suspension by volume) were added to each bottle containing 20 ml. of medium 199 plus 2 per cent calf serum. Bottles were incubated at 37° C. until complete cell sheets were formed over the cover slips, usually for a period of 7 days. At this time the supernatant medium was removed and replaced with fresh medium containing virus inoculum.

Viruses. Adenovirus types 2 through 7 were stock strains maintained in this laboratory. The following average titers were obtained for each type: Type 2, $10^{-2.5}$; Type 3, 10^{-5}; Type 4, 10^{-3}; Type 5,

* Received for publication, November 21, 1597.

10^{-2}; Type 6, 10^{-2}; and Type 7, $10^{-2.5}$. Simian viruses were derived from the original inocula supplied by Dr. R. N. Hull of the Eli Lilly & Co. Research Laboratories, Indianapolis, Indiana. Cells in each bottle were inoculated with 1 ml. of undiluted virus suspension, and the cover slips were removed at various periods of time, depending upon the extent of infection as determined by microscopic examination.

Examination of Cell Sheets. Most specimens were stained with Giemsa stain or hematoxylin and eosin, following fixation in methyl alcohol or 10 per cent formalin. The entire area of each of the cover slips was examined and all obvious cytopathogenic changes recorded. Control slides were prepared for each set of slides from infected cultures.

Results

Infection with Adenoviruses

On the basis of pathologic alterations, we placed the adenovirus types studied into 3 major groups: Group 1 (types 3 and 4) was characterized by nuclear enlargement, eosinophilic granulations, and prominent eosinophilic inclusion bodies. The latter were either homogeneous or exhibited an internal architecture. Group 2 (type 7) was characterized by absence of nuclear enlargement. There were, however, finite intranuclear eosinophilic granulations and inclusions resembling those of group 1. Group 3 (types 2, 5 and 6) was characterized by absence of nuclear enlargement. The inclusions here were basophilic. There were peripheral concentrations of nuclear material, marked shrinkage and distortion of cells, and minimal intranuclear changes.

The report of Boyer and co-workers,[3] which appeared after the present study was undertaken, recorded essentially the same changes in HeLa cells infected with types 3 and 4 adenoviruses as observed by us in monkey renal epithelial cells.

The details of the morphologic structure of the cells infected with adenovirus are indicated below.

Type 2. Enlargement of the nuclei was not a prominent feature, although a large number of cells showed a distorted, irregular outline of the nuclear membranes. The prominent eosinophilic nuclear inclusions noted in HeLa cells[3] were not observed regularly, and some nuclei were shrunken. Basophilic inclusions were present in older infected cells, but were deep-staining consolidated masses similar to those seen in cells infected with types 3 and 4 viruses. Some vacuolization of the cytoplasm was noted, but this and other cytoplasmic changes did not appear to differ from those found in cells infected with other types of adenoviruses.

Type 3. Enlargement of the nuclei was prominent within the first 15 hours after infection, and distortion of nuclear shape with shrinkage occurred later. A large, dark-staining centrally located basophilic body was seen in many nuclei, with a rarefied, almost halo-like zone surrounding it (Figs. 1 and 2). Preceding the formation of the inclusion, numerous granular bodies appeared, tending to cluster and coalesce into masses (Fig. 1). In other cells a granular mass identical with that described in infected HeLa cells was observed. Some cells showed margination of the nuclear substance, central accumulations of eosinophilic material and radiating fibril-like structures (Fig. 3). These are probably similar to the "honeycomb" structures noted in HeLa cells.[3] Some of the cells became segmented and lobulated in appearance, as described by Barski.[1]

Type 4. Most of the changes noted in monkey kidney cells altered by type 3 virus also were evident following infection with the type 4 virus. Many large, central, homogeneous, basophilic, dark-staining bodies occupying 1/3 to 2/3 of the nucleus were seen. Also, in cells containing eosinophilic granulation, the lesion seemed more widely dispersed throughout the nucleus, rather than concentrated in a relatively small central or eccentric position as in the case of the type 3 virus.

Type 5. Nuclear enlargement was not prominent. Margination of nuclear material with rarefaction of the area between the edge of the nucleus and the central inclusions was observed frequently. The central bodies were 1/4 to 1/3 the size of the nucleus and were strongly basophilic. The cells became shrunken, distorted in shape, and finally were lysed or completely lost their morphologic integrity. Large intracytoplasmic nuclei also were seen in many cells. The absence of nuclear enlargement and the type of inclusion development differed distinctly from the features produced by types 3 and 4 virus, but were not unlike those encountered with type 2.

Type 6. Although some nuclei were enlarged, this was not an outstanding characteristic of infection with type 6 adenovirus. Nuclear material tended to concentrate at the periphery of the nucleus, and large centrally located basophilic bodies were similar to those observed in type 5 infection. Many cells showed rarefaction, poor nuclear staining, and were without prominent inclusions. In such cells the nuclei seemed to be vacuolated or contained a lace-like matrix. As the cells approached the advanced stages of degeneration, they became markedly swollen, lost all normal structure, and both nucleoplasm and cytoplasm appeared to become confluent. Large vacuoles appeared in the degenerated cell.

Type 7. This strain of adenovirus did not cause nuclear enlargement. Infected cells exhibited prominence of the nuclear eosinophilic granulations, a feature characteristic of types 3 and 4 virus infections. Some cells showed concentration of the nuclear material at the periphery, but to a lesser degree than occurred in the other types. The central eosinophilic masses had fibril-like connections with the periphery. The microscopic lesion was similar to that encountered in infection with types 3 and 4 virus, but lacked nuclear enlargement. There was also a greater incidence of basophilic concentrations in the nuclei (Fig. 4). These bodies were larger than normal nucleoli and were located centrally, eccentrically or peripherally.

Infection with "Simian Viruses"

Cells infected with some of the "simian viruses" which are common contaminating agents were examined. These agents are designated according to the numerical system outlined by Hull.[5]

SV 17. Enlargement of nuclei was seen in infected cells, and concentrations of eosinophilic granular material were noted in many. An unusual feature was the formation in the nucleus of several cyst-like bodies, each with a central eosinophilic mass surrounded by a clear, light-staining zone. These bodies resembled, in part, those described by Boyer and co-workers[8] in stages 1 and 2, in HeLa cells infected with adenoviruses 1 and 2. When a large mass of eosinophilic granules was present, the nucleolus was displaced to one side and, in some instances, nearly to the periphery of the nucleus. At the same time, the nuclear outline was distorted and occasionally had a beaded appearance. Some cells, apparently in the later stages of infection, were swollen, nuclear outlines were markedly distorted, and deeply stained intranuclear basophilic inclusions were noted.

SV 15. Eosinophilic granulations were present, but were of an entirely different type from those seen in cells infected with the adenovirus. The granules were much larger and were usually distributed throughout the nucleus. As infection progressed, the nucleus enlarged, and the granulations were replaced by large basophilic concentrations that almost filled the entire nucleus. This transition could be seen in some cells as the mass of basophilic material almost seemed to overgrow the granular portions. The infected cells then began to swell, the cytoplasm lost its definition, and the cell degenerated completely. Some cells, however, developed basophilic inclusions without a primary stage of eosinophilic granulation.

SV 11. Only slight changes were seen in cells infected with this

agent. The nuclei were not enlarged, and the only prominent features were pale-staining, basophilic inclusions about 2 to 3 times the size of nucleoli. Infected cells apparently became shrunken, the nuclei elongated, the cytoplasm formed long strands, and finally the entire cell underwent degeneration.

SV 1. Alterations induced by this "simian virus" were similar to those caused by SV 11. The nuclei were not enlarged, but large, pale inclusion bodies were present, in juxtaposition with the nucleoli (Fig. 5).

SV 4. No change in nuclear size was observed, but the nucleoli were markedly enlarged. The cytoplasm was pale-staining and the damage more severe than with other types. Vacuolization of the area adjoining the nucleus was especially pronounced. Pale-staining basophilic inclusions surrounded by rarefied zones also were evident.

SV 16. There was no nuclear enlargement, but numerous cells contained large basophilic granules almost the size of nucleoli. In such cells the cytoplasm also was severely affected, showing a foamy pale-staining character.

SV 18. Large basophilic granulations, similar to those seen in infection were present. The nuclei were shrunken and distorted. There appeared to be an organization of the granules into large, deep-staining, basophilic masses, with no obvious internal structure. The granules appeared to overhang or protrude through the nuclear membrane, giving a beaded appearance to the nuclear periphery. Vacuolization of the cytoplasm was pronounced. In many cells the nucleoli were enlarged and pale staining, as in the lesions produced by the SV 1 and SV 11 strains. Some of the nucleoli had a dark blue, beaded periphery. A tendency to form multinucleated cells was evident in many portions of the sheets in advanced states of infection. There was no nuclear enlargement.

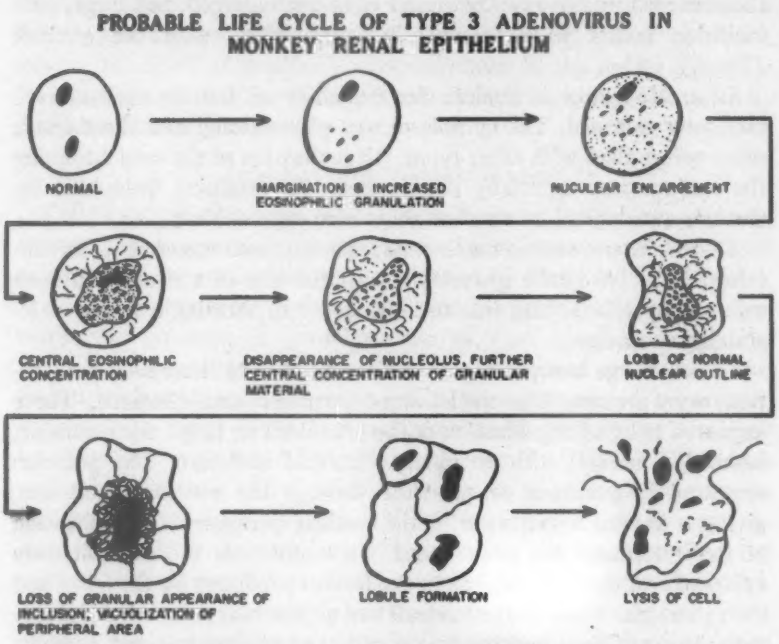
SV 27. The changes produced by this virus were indistinguishable from those induced by adenoviruses types 3 and 4 (Fig. 6).

Discussion

The observations made are inadequate to permit classification of the "simian viruses" on the basis of the cytologic changes reported. Except for the SV 27 virus, the cellular alterations induced by "simian viruses" are distinguishable from those produced by at least 6 types of adenovirus.

The cellular alterations induced by adenoviruses are in the main intranuclear. This has been observed in this and other studies.[7,8,9]

The sequence of nuclear alteration, as determined by studies of tissue culture at varying intervals after virus inoculation, is summarized in Text-figure 1. Although it has been suggested that adenoviruses may multiply without killing the infected cells,[9] our observations tend to indicate that visibly damaged cells proceed to death, and that at least some disintegrate by lysis (Fig. 4).


PROBABLE LIFE CYCLE OF TYPE 3 ADENOVIRUS IN MONKEY RENAL EPITHELIUM
NORMAL
MARGINATION & INCREASED EOSINOPHILIC GRANULATION
NUCULEAR ENLARGEMENT
CENTRAL EOSINOPHILIC CONCENTRATION
DISAPPEARANCE OF NUCLEOLUS, FURTHER CENTRAL CONCENTRATION OF GRANULAR MATERIAL
LOSS OF NORMAL NUCLEAR OUTLINE
LOSS OF GRANULAR APPEARANCE OF INCLUSION; VACUOLIZATION OF PERIPHERAL AREA
LOBULE FORMATION
LYSIS OF CELL

Text-figure 1. The sequence of nuclear alterations induced by adenoviruses, as determined by studies of tissue culture at varying intervals after virus inoculation.

Orphan viruses of simian origin have, in occasional instances, produced lesions in sheets of monkey renal epithelium in tissue culture that resemble, in unstained preparations, those caused by the adenovirus group. Studies of the nuclear changes induced by these agents, however, indicate that of those investigated, only one simian strain, SV 27, causes alterations that are indistinguishable from those produced by the adenoviruses.

Summary

The cellular changes induced in monkey kidney epithelium in tissue culture by strains of adenovirus were investigated. Similar studies were carried out with a group of "simian viruses" and the lesions compared.

Only one "simian virus," SV 27, produced alterations in the nucleus that were indistinguishable from the characteristic lesions of adenoviruses types 3 and 4.

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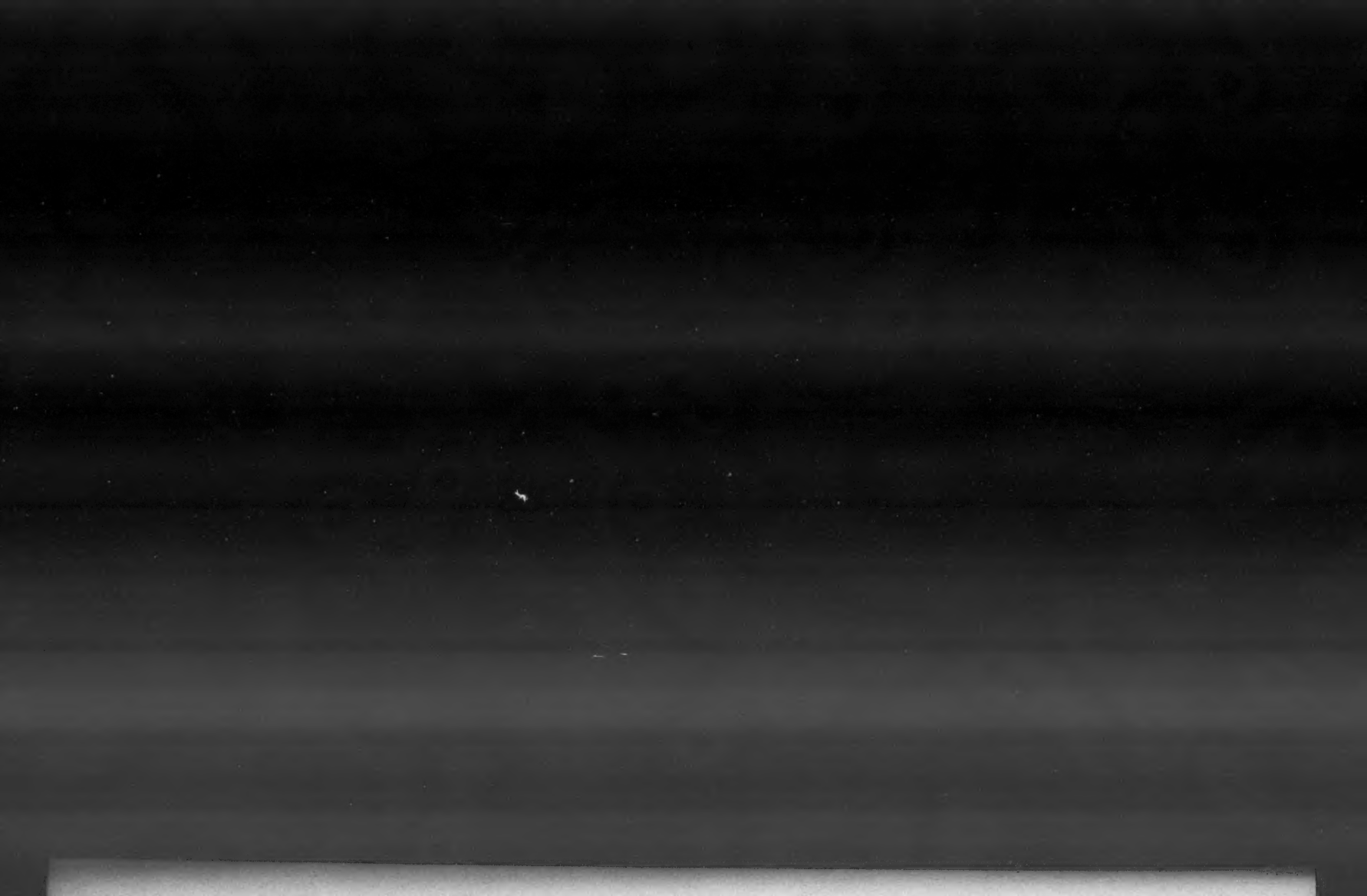
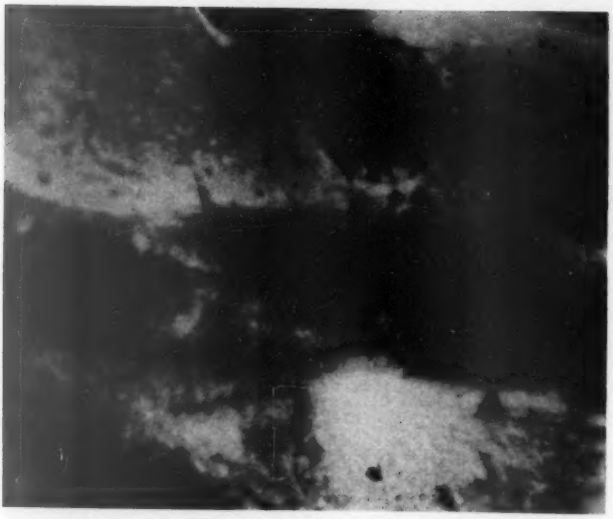
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Adenovirus type 3. Central inclusion (a), accumulations of eosinophilic granular material (b). Giemsa stain. × 1,000.

FIG. 2. Inclusion body, type 3 adenovirus. Giemsa stain. × 1,000.





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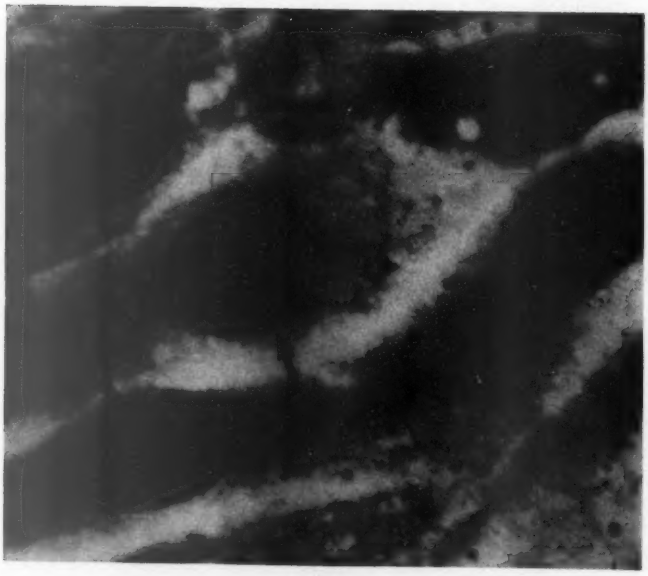

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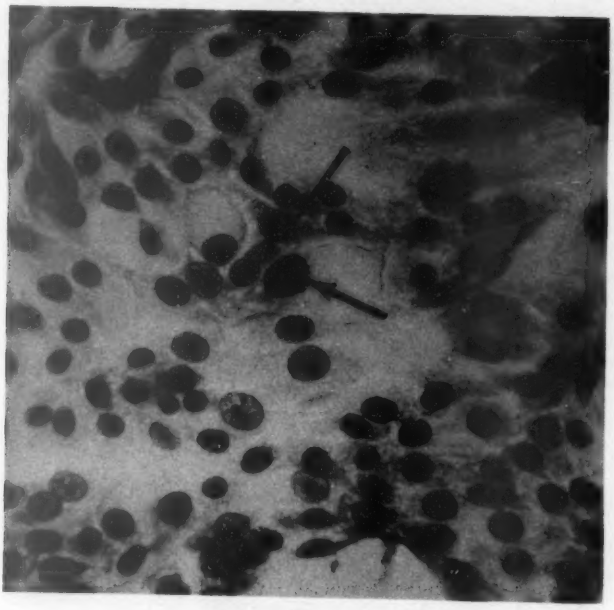
FIG. 3. Inclusion bodies, adenovirus type 3, showing large central mass. Giemsa stain. × 250.

FIG. 4. Central basophilic inclusion bodies of adenovirus type 7. Giemsa stain. × 250.



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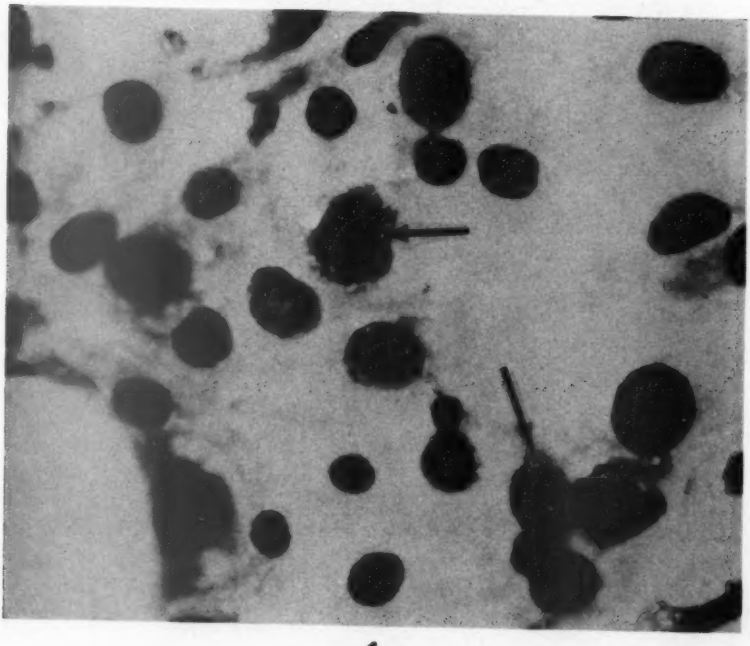

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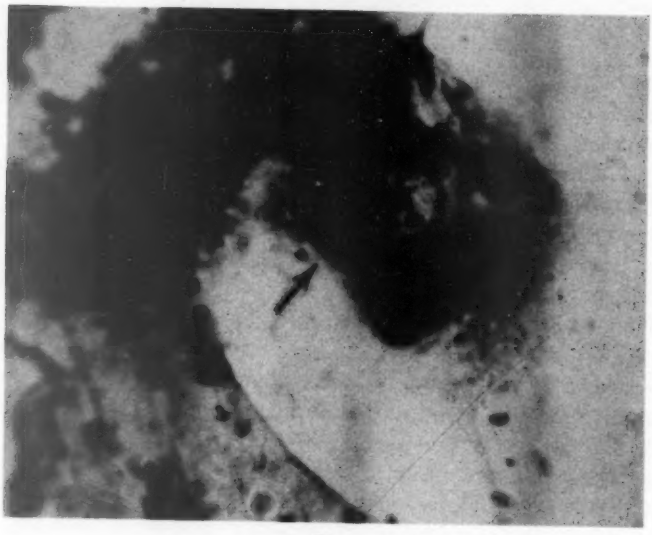
FIG. 5. Monkey kidney epithelial cell affected by simian virus type 1, showing large rarefied areas in the nucleus. Giemsa stain. × 1,000.

FIG. 6. Large inclusion mass, simian virus type 27 (a); inclusion body with surrounding rarefied area (c); normal cell (b). Giemsa stain. × 1,000.



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PATHOGENESIS OF OSGOOD-SCHLATTER'S DISEASE*

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Because of the paucity of material for histologic study, there is considerable confusion in the medical literature concerning the pathogenesis of the lesion in Osgood-Schlatter's disease. In their original papers, Osgood[1] and Schlatter[2] both considered the lesion to result from an avulsion of part of the tibial tuberosity by the indirect trauma of quadriceps muscle contraction. Later the lesion was grouped with Kienböck's disease, Legg-Calvé-Perthes disease, and others in a category called osetochondritis deformans juvenilis. In 1926 Christie[3] wrote: "It is the general opinion that all of these conditions which pass under various names are the same pathologic entity modified only by the particular location in which they occur." The theory offered at that time was that these conditions were all instances of aseptic necrosis of various ossification centers, with subsequent fragmentation of the bone. Judging from many textbooks, this idea is still prevalent. In 1930 Phemister and his associates[4] reported culturing *Streptococcus viridans* from lesions of Köhler's, Legg-Perthes, and Kienböck's diseases, and in 1934 Pease[5] reported the isolation of similar organisms from lesions of Osgood-Schlatter's disease. In 1937, Cole[6] proposed that the disorder was primarily a degeneration of the tendon due to strain near its insertion, with formation of ossicles by metaplasia in the tendinous tissue. Uhry[7] reported the pathologic anatomy of Osgood-Schlatter's disease in 1944, and concluded that trauma to the epiphysis could explain all the findings. A clinical resumé of the disease was presented by Kridelbaugh and Wyman in 1948[8]; they emphasized that the condition was more common than was generally appreciated.

Nine specimens from the tibial tuberosities of 7 cases of Osgood-Schlatter's disease form the basis of this report. In 2 cases the lesions were bilateral. All of the patients were males, ranging in age from 13 to 17 years. At the time of operation, an attempt was made to excise an intact block of tissue including tendon, ossicle, and underlying bone. The tissue was decalcified, and microscopic sections were prepared in a sagittal plane through the entire lesion. Sections were stained with hematoxlyin and eosin, and examined under ordinary and polarized light.

* Received for publication, December 18, 1957.
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Ten additional cases were studied, in which only a separate ossicle in the tendon was available for histologic examination. These ossicles were found to consist of bone and cartilage, with callus formation, focal aseptic necrosis, and degenerative changes in the neighboring tendinous tissue. There was no evidence of osteomyelitis in any of these. Because the specimens were incomplete, no positive conclusions were drawn from them.

Report of Cases

The clinical features of these cases are assembled in Table I.

Case 1

Roentgenograms made after an acute exacerbation showed that the epiphyseal line was normal and open (Fig. 1). The anterior surface of the tibial tuberosity was irregular, and a separate fragment was noted within the patellar tendon a few millimeters away. There was swelling of the tendon and surrounding soft tissue.

Histologic Observations. A thin, even layer of cartilage with evidence of endochondral ossification was still present along the anterior surface of the tuberosity, and the heavy collagen fibers of the tendon inserted through this thin layer into the cortex of the tuberosity. There was, at one point, a definite defect in the anterior cortex of the tuberosity, filled with highly cellular and very vascular granulation tissue (Fig. 2). The separate ossicle in the tendon seemed to fit into this defect in the tuberosity like a piece of a jigsaw puzzle. Examination under polarized light emphasized the defect (Fig. 3). There was no evidence of recent or old hemorrhage. The broken spicules of the tuberosity itself were viable and covered by thick layers of osteoid with prominent osteoblasts (Fig. 4). In contrast to the tuberosity, the bone of the separate ossicle was mostly necrotic, though the marrow was viable and composed of cellular fibrous tissue. There was active osteoid apposition about some of the necrotic spicules (Fig. 5), and numerous osteoclasts were present near these fragments and in the subjacent granulation tissue.

Case 2

A roentgenogram revealed swelling of the patellar tendon, with separate, small, rounded ossicles in the tendon near its insertion. The epiphyseal plate was closed. After 10 months of conservative management with no response, an operation was performed. The block of tissue removed included the insertion of the patellar tendon, the anterior portion of the tibial tuberosity, and the ossicle within the tendon.

Histologic Observations. All the bone tissue of the tibial tuberosity contained well preserved osteocytes (Fig. 6). A small defect measuring up to 2 mm. in width was found in the cortical bone of the tuberosity along the line of insertion of the tendon. The defect was filled with moderately vascularized, dense collagenous connective tissue

TABLE I
Clinical Data

Case no.	Age	Sex	Race	Side affected	Symptoms	Duration of symptoms at time of operation
1	14	M	W	L	One year history of vague discomfort about knee on exercise. Acute exacerbation of pain and swelling over tibial tuberosity followed jumping and landing heavily on feet while playing basketball.	16 days
2	14	M	W	R	Pain and swelling over tibial tuberosity after running. No clear history of trauma.	Indefinite; about 2 years
3	13	M	W	R	Patient tripped and fell to knees; experienced painful swelling of tibial tuberosity on exercise thereafter.	5 months
4	16	M	N	Bil.	Pain and swelling over tibial tuberosity on exertion. No clear history of trauma.	Indefinite; about 2 years
5	13	M	W	R	Symptoms began after "bumping" knee several times while playing football.	7 months
6	17	M	W	Bil.	Pain and swelling over tibial tuberosity on exertion. No clear history of trauma.	Indefinite; about 4 years
7	15	M	W	L	Pain and swelling over tibial tuberosity on exertion. No clear history of trauma.	2 to 3 years

which flowed through the cortical bone and into the marrow spaces of the underlying cancellous bone. The neighboring spicules were irregular, surrounded by thick zones of osteoid and prominent osteoblasts. A considerable amount of callus was present at the margins of the defect. Just between this defect and the ossicle in the tendon was a zone of fibrous tissue and fibrocartilage, with an irregular cleftlike space partly filled with fibrillary, basophilic material and small, irregular fragments of calcified material resembling splinters of bone or calcified cartilage. The surface of the ossicle facing the underlying bone was covered by callus. The opposite surface of the ossicle, closest to the patella (Fig. 7), was relatively regular, showing an orderly insertion of the tendinous fibers into a smooth row of cortical bone. The latter showed only slight osteoblastic proliferation, and there was no callus in this region.

Case 3

Roentgenograms showed swelling of the tendon over the tuberosity and irregularity of the anterior surface of the tuberosity, with several small separate ossicles (Fig. 8). Under general anesthesia a block of tissue including the anterior portion of the tibial tuberosity and a segment of the tendon was removed.

Histologic Observations. The specimen included the entire lower tip of the tibial tuberosity, the epiphyseal plate, and a thin layer of the underlying metaphysis (Fig. 9). The line of insertion of the tendon into the anterior surface of the epiphysis was irregular, with one large defect filled with callus and surrounded by bony spicules with broad osteoid seams covered by plump osteoblasts. There was fibrosis of the marrow and no evidence of necrosis in the tuberosity itself. A small, separate, necrotic chip of bone was embedded in the connective tissue next to the defect in the surface of the tuberosity, but most of the intratendinous ossicles were not included in the specimen. In the epiphyseal plate, the cartilage contained foci of irregular proliferation, characterized by clusters of large chondrocytes arranged in small balls and whorls. Near the distal tip of the tubercle, where the epiphyseal plate blended with the tendinous tissue, the matrix of the cartilage showed focal cystic mucoid degeneration. There was no evidence of necrosis in the cartilage.

In this case the separate ossicles observed in the tendon in the roentgenograms were not removed for histologic study, but the defect in the underlying tibial tuberosity with callus around it suggested that a small fragment of bone had been torn away. The foci of cystic mucoid degeneration without necrosis in the epiphyseal cartilage were not considered to be of primary importance.

Case 4

Roentgenograms of the right leg showed irregularity of the anterior surface of the tibial tuberosities and separate irregular bodies in the tendon. A block of tissue including the tendon and anterior surface of the tuberosity was prepared from each side. The epiphyseal line was normal and open. Postoperative films on the right showed that the tissue removed included the inferior tip of the tuberosity with a portion of the underlying epiphyseal plate. Most of the separate calcified bodies remained in the tendon above the operative site. No definite intratendinous ossicles were visible on the left. Postoperatively, the patient suffered a prolonged period of disability with swelling and pain in the operative sites.

Histologic Observations. Sections from the right tibia included a large segment of the epiphyseal plate with the underlying metaphysis. There was also a large portion of the tibial tuberosity with attached tendon. A few small intratendinous fragments of bone and cartilage were also included, but the larger ossicles observed in the roentgenograms were not in the specimen. The epiphyseal line was orderly and the cartilage of the epiphyseal plate well preserved except for minute foci of mucoid degeneration. No lesions were found in the metaphysis. The tuberosity showed an irregular anterior surface along which there was marked osteoblastic activity with osteoid apposition, and small foci exhibiting irregular proliferation of cartilage and transformation into bone (callus). The neighboring marrow spaces were filled with plump fibroblasts and newly formed vessels. In the extra-osseous fibrous tissue adjacent to the anterior cortical surface there were a number of irregular spicules of necrotic bone and callus, some surrounded by foci of cystic mucoid degeneration.

The specimen from the left side included the normal epiphyseal plate and ossification center of the tuberosity, but neither a defect in the tuberosity, nor bony fragments in the tendon could be demonstrated.

Case 5

The roentgen films were not available.

Histologic Observations. The epiphyseal plate of cartilage was broad and well preserved, except for scattered minute lesions exhibiting fibrillary alteration, cyst formation, and calcification (Fig. 10). Chondrocytes surrounding these foci were somewhat more numerous than elsewhere, and tended to form a cellular rim about the areas of altered matrix. There was no evidence of necrosis or cleavage in the cartilaginous plate. The bony epiphysis of the tuberosity showed a large defect bordered by callus in its anterior cortex. Polarized light failed to demonstrate refractile collagen in this region (Fig. 11). The intact portion of the anterior cortex of the epiphysis was covered by a

thick zone of hyaline cartilage into which fibers of the patellar tendon inserted. The separate ossicle was not included in the specimen.

Case 6

Roentgenograms showed slight soft tissue swelling over both tibial tuberosities. The epiphyses were completely closed. A large ossicle was noted on each side in the patellar ligament near its insertion; the one on the right appeared to be fragmented. On the left, the ossicle had an intact delicate rim of cortical bone with a cancellous structure in the center. A block of tissue including the ossicles, a segment of tendon, and the anterior portion of the tibial tuberosity, was removed from each knee.

Histologic Observations. The tissue from the right knee included a large portion of the anterior cortex of the tuberosity. This was intact except for a few small (1 to 2 mm.) focal cortical defects filled with cellular vascularized fibrous tissue. These were surrounded by spicules which showed active osteoblastic proliferation and osteoid apposition. The cortical bone near the defects was exceptionally thick, in contrast to the delicate layer of cortical bone and the slender bony spicules at the upper margin of the specimen. The ossicles in the tendon were relatively mature but the margin closest to the underlying tuberosity showed evidence of growth, characterized by the apposition of osteoid to bony spicules and the proliferation of cartilage with direct transformation into bone. A relatively large amount of callus was found about the smaller of the two ossicles. The tissue between the ossicles and the underlying bone was composed of dense cartilaginous tissue and fibrocartilage with somewhat irregularly arranged fibers. Several irregular cleftlike spaces resembling pseudarthroses were noted in the fibrocartilage.

The lesions in the specimen from the left knee were essentially similar to those on the right except that a definite defect in the cortical bone was not demonstrated.

Case 7

Roentgenograms showed a normal, unfused, but narrow, epiphyseal plate, irregularity of the anterior surface of the tibial tuberosity, and small separate densities in the tendon. The tendon was greatly swollen in this region. At operation the block of tissue removed included the areas of increased density in the tendon, the insertion of the tendon, underlying epiphyseal bone, a portion of epiphyseal plate, and a narrow margin of metaphysis.

Histologic Observations. The metaphysis was not remarkable, and the epiphyseal plate was normal, with active endochondral bone formation. The portion of the bony epiphysis next to the epiphyseal plate was intact, containing regularly arranged spicules lined by flattened osteoblasts. In contrast to this, the anterior superior surface of the tuberosity was irregular, and the cortical bone exhibited small defects filled with cellular and vascularized connective tissue and callus. Bone

spicules surrounding these regions were covered by thick osteoid seams and prominent osteoblasts. The rest of the anterior surface of the tuberosity was covered by a thin layer of hyaline cartilage into which fibers of tendinous tissue inserted. The intratendinous ossicle closer to the tuberosity lay next to the defect in the cortex of the tuberosity. Part of the ossicle was composed of regular cortical bone covered by an orderly layer of cartilage similar to that found along the cortical surface of the tuberosity itself. In contrast, other parts of the ossicle were irregular, with jutting ends of focally necrotic bony trabeculae surrounded by cellular fibrous tissue. The second ossicle had a similar histologic appearance, except that it contained more cartilage, and no really orderly architecture was evident. The tissue between the two ossicles consisted of fibrocartilage, containing several large cleftlike spaces lined by fibrillary eosinophilic material resembling fibrin, and small fragments of necrotic bone and cartilage.

Discussion

A defect in the anterior cortical bone of the tibial tuberosity was found in every case. A zone of proliferating connective tissue with differentiation into irregularly arranged osteoid tissue, and occasionally cartilage, filled and surrounded the defect. The neighboring bone spicules showed osteoblastic proliferation with osteoid apposition, features characteristic of callus. In no case was there evidence of necrosis in either the epiphyseal bone or the epiphyseal plate. There was no evidence of suppuration. The size of the defects in the tuberosities varied considerably. In the case with the shortest duration (case 1), the defect was the same size as the ossicle, which seemed to fit into the defect (Figs. 2 and 3). In other cases the defect was smaller than the ossicle, presumably having been partly filled by the proliferation of bone at the margins.

The ossicle in the tendon consisted of bone surrounded by callus, with areas of necrosis in some cases. In cases 1 and 2, the surface of the ossicle nearer the patella was characterized by an orderly layer of cortical bone, without evidence of callus or osteoid apposition (Fig. 7). The fibers of the patellar tendon inserted in a regular fashion into this portion of the ossicle, suggesting that it originally had formed part of the normal cortex of the epiphyseal bone, and had been displaced. This interpretation is further supported by the irregular surface covered by callus on the side of the ossicle facing the tibia. In some cases a cleft resembling a pseudarthrosis was found in the fibrocartilaginous tissue between the tuberosity and the ossicle, or between two ossicles. The tendinous tissue about the ossicle frequently showed

increased vascularity, and occasionally contained foci of mucoid degeneration.

The epiphyseal plate did not seem to be involved primarily in these cases. There was evidence of neither necrosis nor a line of cleavage through the cartilage, and scarring, indicative of an old healed defect in this region, was lacking. The foci of mucoid degeneration and the occasional nests of irregularly proliferating chondrocytes were small and not considered of contributory significance.

Visualization with polarized light emphasized the defect along the anterior surface of the tuberosity. In normal tissues the dense collagen fibers of the tendon were found to interlock with those of the bone, forming a continous pattern of highly refractile tissue. When a fragment was found to be separated, the defect was filled with young cellular fibrous tissue. With polarized light, because of the greater refractility of the dense collagen fibers of the tendon and bone, the difference between this collagen-poor tissue and normal tendon became apparent. The gap in a relatively early case (Fig. 3) was clearly delineated. In cases of longer standing, granulation tissue lay down new collagen, forming a scar, and partially filled the defect in the refractile tissue.

From these findings it was concluded that the intratendinous ossicle in Osgood-Schlatter's disease was derived originally from an avulsed fragment of the tibial tuberosity. This was best shown in the case of short duration (case 1). Later the ossicle was enlarged by the proliferation of callus about it; the defect in the cortex of the tibial tuberosity was gradually filled by scar and callus. The ossicle might be fractured, forming two or more ossicles (*os bipartitum* or *tripartitum*). A cleft resembling a pseudarthrosis occasionally developed in the fibrocartilage between the several ossicles or between the ossicle and underlying bone. The avulsed fragment was occasionally necrotic and, in some instances, replaced by creeping substitution. No evidence of primary necrosis or inflammation was found in either the cartilage or bone of the tibial tuberosity itself. Since there was no evidence of a primary disorder of the affected tissue, the best explanation for the pathogenesis of the condition was that the force of quadriceps contraction concentrated on a small portion of incompletely developed bone produced an avulsion fracture. Osgood, in his original article[1] demonstrated the mechanism of this type of fracture by means of dissection and manipulation of cadavers. Our findings support his original views, except that in our material the line of separation of the fragments occurred not through the cartilaginous plate, but through the ossification center itself.

Summary

The histologic alterations in 9 specimens from the tibial tuberosities of patients with Osgood-Schlatter's disease are presented.

Evidence indicates that the ossicle in the tendon was formed initially by avulsion of a portion of the tibial tuberosity. The avulsed fragment became necrotic, and creeping substitution, remodeling and enlargement ensued.

No evidence of inflammation or primary aseptic necrosis was found in the tibial tuberosity of any of the cases.

Osgood-Schlatter's disease is believed to be of traumatic origin and is attributed to the pull of the quadriceps tendon on the apex of the bony tuberosity.

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The authors wish to express their appreciation to Dr. Paul Kimmelstiel, Chief of the Department of Pathology of the Charlotte Memorial Hospital, for his encouragement and advice in the preparation of this paper.

[*Illustrations follow*]

LEGENDS FOR FIGURES

All microscopic sections stained with hematoxylin and eosin stain.

FIG. 1. Case 1. Preoperative roentgenogram showing ossicle, with site of biopsy outlined in black.

FIG. 2. Case 1. Photomicrograph of zone outlined in the roentgenogram. The small rectangles indicate the fields shown in higher magnification in Figures 4 and 5. × 6.

FIG. 3. Case 1. Area shown in Figure 2, as seen with polarized light.

FIG. 4. Case 1. Higher magnification of the spicule of bone outlined on the left side of Figure 2. Note well preserved osteocytes and osteoblastic proliferation. × 80.

FIG. 5. Case 1. Higher magnification of bone from avulsed fragment, outlined in the rectangle to the right in Figure 2. Note poorly stained osteocytes in necrotic spicules, and beginning osteoid formation at the end of the spicule in the center of the field. × 80.

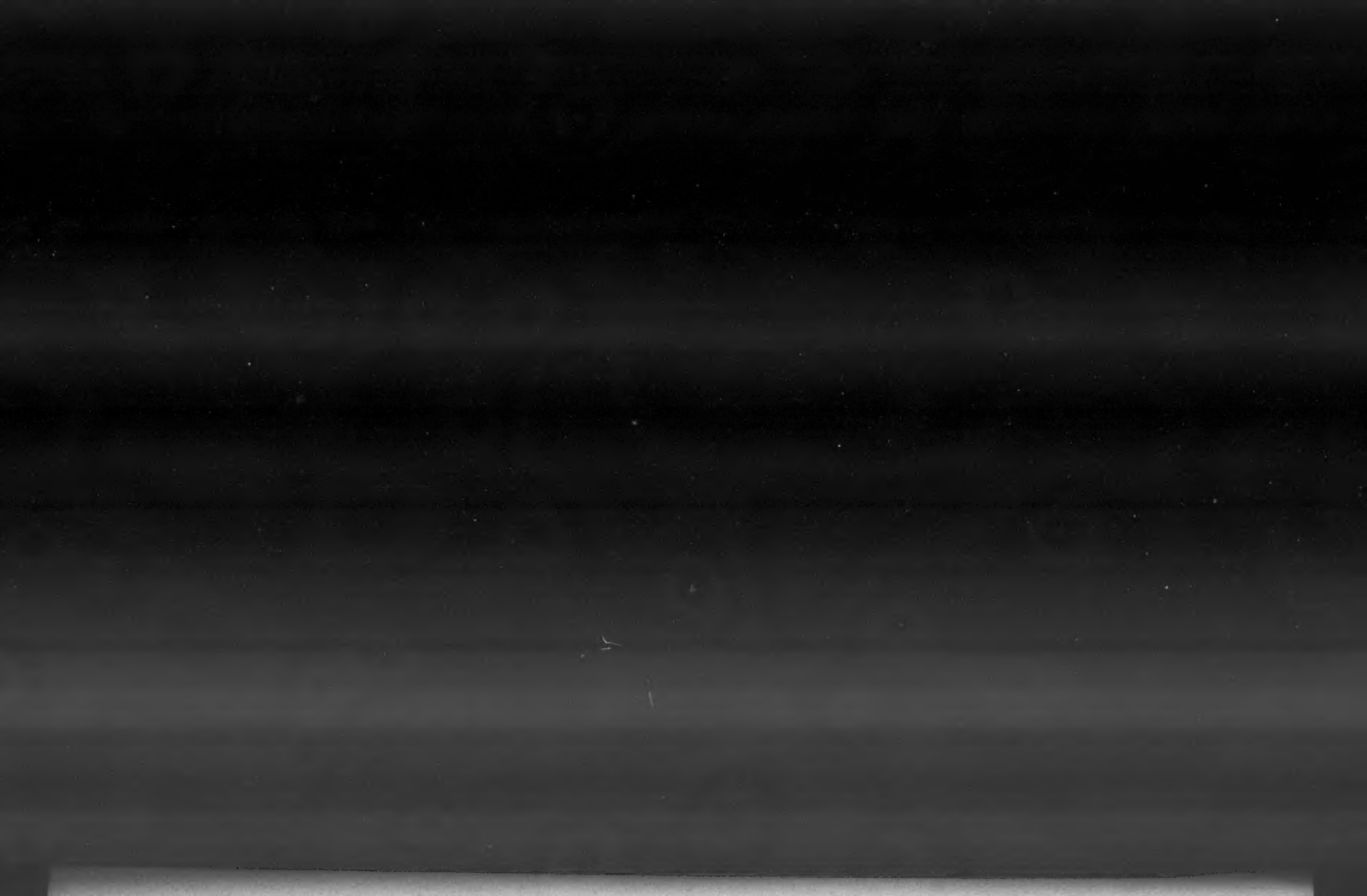

FIG. 6. Case 2. Orientation similar to that shown in Figure 2. × 6.

FIG. 7. Case 2. Higher power view of area outlined in Figure 6, showing orderly insertion of patellar tendon into cortical bone. × 80.

FIG. 8. Case 3. Roentgenogram of affected knee, with site of biopsy outlined in black. Note that ossicles are not included in this biopsy specimen.

FIG. 9. Case 3. Low power magnification of area indicated in Figure 8. The epiphyseal plate is at the left of the photograph; the bone constitutes the entire tip of the beak-like ossification center of the tuberosity. Note the large defect in the bone near the upper margin of the field, filled with connective tissue. The epiphyseal plate shows irregular proliferation of chondrocytes. × 5.

FIG. 10. Case 5. Orientation of photograph similar to Figure 9. Note defect in the tuberosity. × 5.

FIG. 11. Case 5. Same field shown in Figure 10, examined under polarized light. Note lack of refractile tendinous tissue in the defect.

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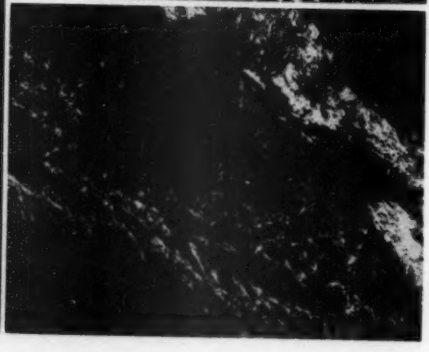

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